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Regulation of Neutrophilic Inflammation by Hypoxic Signalling Pathways

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I. Abstract

Neutrophils are essential for effective innate immunity. Conversely, inappropriate or excessive neutrophil activation can result in damaging inflammation. This damage is implicated in the pathogenesis of a number of respiratory diseases including acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD) which are also both frequently complicated by hypoxia. Cells sense and respond to hypoxia through the activity of the transcription factor HIF (hypoxia inducible factor) and its regulatory hydroxylases, the prolyl hydroxylase domain enzymes (PHDs) 1-3. In the presence of oxygen, PHDs hydroxylate HIF, preventing the HIF mediated transcriptional response. Close links exist between the pathways which regulate hypoxic and inflammatory responses. Our group has previously found that in mouse models of infection, acute hypoxia leads to increased sickness and that this is driven by neutrophilic inflammation.

I have used a murine model of Lipopolysaccharide (LPS) -induced acute lung injury, characterised by neutrophil influx, to investigate how exposure to hypoxia alters lung inflammation. Using high-resolution mass spectrometry, I have defined the proteome of the inflammatory lung neutrophil. I have shown that hypoxia results in a distinct proteomic signature in inflammatory neutrophils. Hypoxia drives lung neutrophilic inflammation through increased neutrophil degranulation and upregulation of inflammatory receptors.

I have also identified key metabolic alterations in hypoxic neutrophils. The hypoxic lung represents a low glucose, high protein environment and neutrophils adapt to exploit this. I have shown that neutrophils can scavenge

proteins from their extracellular environment, catabolise these proteins in the lysosome and utilise the breakdown products for metabolism. These processes are upregulated in hypoxic lung neutrophils which show increased lysosomal protein expression, increased protein uptake and increased glutaminolysis. Utilising heavy labelled protein extracts, I have traced breakdown products from scavenged proteins into central carbon metabolism, demonstrating that extracellular protein can fuel neutrophilic inflammation.

Finally, I have investigated the role of the prolyl hydroxylase PHD1 in regulating neutrophilic inflammation. Using a neutrophil specific PHD1 knockout mouse line, I have identified a specific role for PHD1 in regulating neutrophil metabolism and survival. I have found that the microenvironment, particularly oxygen availability, determines the impact of PHD1 loss with consequences for inflammation resolution *in vivo*.

In summary, hypoxia is a key regulator of neutrophil function and is associated with increased neutrophilic inflammation. Utilising a proteomic approach, I have identified the mechanisms which drive the hyperinflammatory phenotype including the ability of neutrophils to scavenge proteins from the environment to fuel inflammation. I have also shown that PHD1, a key component of the hypoxic signalling pathway, may regulate these functions. A more complete understanding of these mechanisms will help to identify therapeutic targets for treatment of neutrophilic inflammation in the lung.

II. Lay Summary

Neutrophils are a subset of white blood cells which act as the first responders to infection or injury. However, they can also damage our own tissues and inappropriate neutrophil recruitment or persistence of neutrophils after the risk of infection has passed can cause disease. This is true in the lung where neutrophils are associated with both acute illnesses (such as acute lung injury) and chronic diseases (such as smoking related lung disease). Areas of infection or injury often have low oxygen levels and low nutrient levels and neutrophils are well adapted to function in this environment. Additionally, low oxygen levels alter the way neutrophils behave and may increase their capacity to cause tissue damage. The aim of this project was to understand how low oxygen levels change the behaviour of neutrophils and how this may lead to more severe illness.

In order to address this, I used a mouse model of acute lung injury (ALI) which is known to cause neutrophils to move into the airways and cause lung damage. I compared mice which had ALI alone with mice which had ALI and were also exposed to low oxygen levels. I used proteomics to find differences between the lung neutrophils from these two groups. Proteomics describes a technique where all of the proteins in a sample of cells are extracted and analysed to show how much of each protein there is (the proteome of a cell). Computer software is then used to find patterns in the data which shed light on the important pathways and processes which explain the differences we see.

I have shown that in low oxygen levels, mice with an acute lung injury become sicker and develop more severe lung damage than the mice in normal oxygen levels. I have found that there are many differences between the proteomes of neutrophils from the two different groups. An important difference is that neutrophils from the low oxygen mice showed higher levels of receptors which transmit inflammatory signals,

suggesting they are more damaging to the lung than neutrophils in normal oxygen levels. I have also shown for the first time that, when there are low levels of sugar to use as fuel, as is the case in the lung, neutrophils can adapt their behaviour accordingly. They switch on pathways which allow them to take up protein from the environment and they can break down this protein and use it as fuel for energy. Finally, I have shown that by targeting a specific protein (PHD1) which is part of the machinery which allows cells to respond to low oxygen levels, we can alter neutrophil behaviour and response to low oxygen levels, resulting in more rapid recovery of the lung following acute lung injury in low oxygen conditions.

These findings have important implications for human lung diseases. Treatments which stop neutrophil mediated tissue damage have been difficult to develop because, without neutrophils, we are vulnerable to very serious infections. By better understanding how neutrophils behave in the context of an acute inflammatory response, as seen in the acute lung injury model, we aim to develop more specific therapies which could restore normal neutrophil function, without switching it off altogether. The data in this thesis has identified a novel neutrophil behaviour, namely using proteins to fuel inflammation, and further research will aim to find ways to target this process to treat neutrophilic lung damage.

III. Declaration

I declare that the content of this thesis is my own work and that all contributions and collaborations have been explicitly acknowledged in the text. No material presented in thesis has been submitted to any other university or for any other degree.

Emily R Watts

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IV. Acknowledgements

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V. Abbreviations

ALI – Acute lung injury
AMPK – AMP activated protein kinase
ARDS – Acute respiratory distress syndrome
CF – Cystic fibrosis
CGD – Chronic granulomatous disease
CLR – C-type lectin receptor
COPD – Chronic obstructive pulmonary disease
DAMP – Damage associated molecular pattern
FACS – Fluorescence activated cell sorting
FIH – Factor inhibiting HIF
fMLF - Formylated Met-Leu-Phe
FPR – Formylated peptide receptor
GM-CSF – Granulocyte-macrophage colony-stimulating factor
GMP – Granulocyte macrophage precursor
HIF – Hypoxia inducible factor
HRE – Hypoxia response element
IFN-γ - Interferon-γ
IP - Intraperitoneal
LAD – Leucocyte adhesion deficiency
LDHA – Lactate dehydrogenase A
LPS - Lipopolysaccharide
MMP9 – Matrix metalloproteinase 9
MOI – Multiplicity of infection
MPO – Myeloperoxidase
mROS – Mitochondrial reactive oxygen species
mTOR – Mammalian target of rapamycin
NET – Neutrophil extracellular trap
NLR – Nod like receptor
ODDD – Oxygen dependent degradation domain
OxPPP – Oxidative pentose phosphate pathway
PAF – Platelet activating factor
PAMP – Pathogen associated molecular pattern
PHD – Prolyl hydroxylase
PRR – Pattern recognition receptor
ROS – Reactive oxygen species
TLR – Toll-like receptor
TNF-α – Tumour necrosis factor-α

Chapter 1: Introduction

The neutrophil in health and disease

Neutrophils are essential mediators of the innate immune response, without which we are subject to overwhelming infection. This is evidenced by a number of primary immunodeficiencies, such as chronic granulomatous disease (CGD) and leucocyte adhesion deficiency (LAD), characterised by defective neutrophil function which result in susceptibility to life-threatening bacterial and fungal infections[1].

Neutrophils are the most abundant circulating leucocyte. They are derived from granulocyte/macrophage precursors (GMPs) in the bone marrow and are released into the circulation as mature, terminally differentiated cells. They have a relatively short half-life in the circulation, usually in the order of hours. However, *in vivo* labelling experiments have shown that neutrophils may persist for up to 5 days in the circulation[2]. There is also a large pool of neutrophils 'in reserve' in the bone marrow which can be rapidly mobilised to the circulation in response to inflammatory or infective stimuli[3]. Likewise, circulating blood neutrophils can rapidly migrate to injured tissue. Neutrophils are the first cells of the innate immune system to arrive at a site of infection and have an arsenal of antimicrobial functions which serve to recognise, control and eradicate infectious agents.

Neutrophil homeostasis

Neutrophil numbers are regulated by a variety of mechanisms. These include the rate of production, release from the bone marrow and removal by cell death in the periphery or following return to the bone marrow. Chemokines play an

important role in this homeostasis. They are small molecules with characteristic conserved cysteine residues, subdivided based on the position of these residues (CXC and CC chemokines). Their receptors are seven transmembrane G-protein coupled receptors whose downstream signalling includes activation of PI3-kinase, required for chemotaxis. Chemokine signals are essential in regulating neutrophil release from the bone marrow. CXCL12 signalling through the receptor CXCR4 is a strong retention signal for neutrophils in the bone marrow[4]. Conversely, CXCR2 expression on bone marrow neutrophils mediates mobilisation of neutrophils from the marrow in response to ligands including granulocyte colony-stimulating factor (G-CSF)[5]. Furthermore, upregulation of CXCR4 can aid homing to the bone marrow for clearance. This phenomenon has been recognised in both senescent circulating neutrophils[6] and in inflammatory neutrophils during resolution of inflammation in the liver[7]. Chemokines also play an important part in recruitment of neutrophils from the circulation to the site of injury. They are released by cytokine activated endothelial and epithelial cells, tissue resident cells and other leukocytes in injury or infection, leading to neutrophil chemotaxis. This response is then amplified as neutrophils themselves secrete further chemokines to recruit further effectors of the immune response.

Pathogen and damage recognition pathways

Unlike the lymphocytes of the adaptive immune system, neutrophils do not recognise specific pathogens but possess a number of pattern recognition receptors (PRRs) which can sense both microbial motifs (pathogen associated molecular patterns, PAMPs) and ‘self’ danger signals known as danger

associated molecular patterns (DAMPs). Infective and sterile tissue injury will result in the generation of DAMPs, predominantly from necrotic cells. DAMPs include ATP, heat shock proteins (HSP), mitochondrial DNA and formylated peptides and nucleic acids. The presence of DAMPs in the microenvironment has a significant impact on the innate immune response, creating microenvironmental danger signals.

Extracellular ATP enhances neutrophil recruitment, although the mechanism is complex and not a simple chemotactic signal. Chemotaxis is amplified by neutrophil release of ATP in response to activating stimuli, and ATP acts in an autocrine fashion to ligate the G-protein coupled $P2Y_2$ receptor to control orientation[8]. Mitochondrial derived peptides also act as DAMPs. They are formylated peptides which resemble bacterial peptides and signal via the same formylated peptide receptors (FPRs, discussed below) to mediate neutrophil chemotaxis and activation. A hierarchy of processes is proposed to control neutrophil chemotaxis in response to DAMPs and mitochondrial formylated peptides are found to be crucial for the last stages of migration[9].

Neutrophils express multiple PRR subtypes which recognise PAMPs including Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and NOD-like receptors (NLRs). TLRs 1,2 and 4 recognise components of the bacterial cell wall while TLR7 (in mice) and TLR8 (in humans) recognise viral single stranded RNA. Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria, it is a well described TLR-4 agonist and is used throughout this thesis as an inflammatory stimulus, both *in vivo* and *in vitro*. Neutrophils also express multiple CLRs which recognise carbohydrate

moieties. The CLRs Dectin-1 and 2 as well as Mincle recognise fungal cell wall components while MDL-1 recognises flaviviruses such as the Dengue virus. CLRs are cell surface receptors while TLRs can be expressed either on the cell surface or on the endolysosome membrane. NOD like receptors are cytoplasmic PRRs and can be broadly divided into two subgroups: NOD1 and NOD2 recognise components of peptidoglycan in the bacterial cell wall and downstream signalling results in neutrophil activation while the NLRP receptors form an inflammasome complex[10,11]. Finally, formylated peptide receptors (FPRs) are a family of G-protein coupled receptors which recognise both bacterial and mitochondrial formylated peptides. Neutrophils express both FPR1 and FPR2 and activation of these receptors stimulates multiple neutrophil functions including chemotaxis, degranulation, and the respiratory burst[12]. Through this repertoire of receptors neutrophils recognise and respond to a wide range of pathogens. More recently, a zebrafish model has shown that neutrophils are only recruited in response to PAMPs when there are concurrent DAMP signals, suggesting a potential mechanism for innate immune tolerance of non-pathogenic infectious agents[13].

DAMPs and PAMPs, combined with chemoattractant signals and adhesion molecules on the inflamed endothelium combine to recruit neutrophils to the area of injury. Neutrophil migration in response to these signals depends upon expression of receptors and ligands on the surface of both the endothelium and on the neutrophil itself and is discussed in further detail in chapter 3.

Phagocytosis

Upon recruitment and pathogen recognition, neutrophils begin to target the infection. Along with macrophages, neutrophils are termed the professional phagocytes. Phagocytosis may occur in response to PRR ligation or binding of opsonised pathogens to Fcγ receptors. Irrespective of the upstream signals, activation of these receptors results in intracellular signalling cascades involving PI3K and Phospholipase C(PLC) [14] which ultimately lead to actin cytoskeletal rearrangement and formation of a phagosome. Phagosomal maturation ensues, resulting in fusion of the phagosome with intracellular granules present in neutrophils. These organelles possess multiple antimicrobial proteases and also recruit antimicrobial enzymes such as the NADPH oxidase which produces reactive oxygen species (ROS) [15]. These combined mechanisms result in effective intracellular pathogen killing. In addition to these intracellular killing mechanisms, neutrophils may also kill extracellular pathogens through the release of antimicrobial components via the process of degranulation which will be discussed in detail in chapter 3.

Respiratory burst

The neutrophil respiratory burst describes the increased oxygen consumption which accompanies phagocytosis of pathogens. The respiratory burst occurs due to the increased generation of reactive oxygen species (ROS) by the NADPH oxidase, a multicomponent enzyme which generates ROS through electron transfer from cytosolic NADPH to O_2 within the membrane bound organelle[16]. Upon activation, the cytosolic components of the oxidase translocate to join the membrane bound b-type cytochrome component found

in neutrophil granules, thus forming a functional electron transfer system. ROS are highly toxic to invading pathogens and the ROS containing granules may fuse with the phagosome, facilitating intracellular killing or may be released upon degranulation. The critical importance of the NADPH oxidase is highlighted by the overwhelming infections that characterise the clinical course of patients with CGD, in which there is a mutation in one of the components of the NADPH oxidase. Neutrophils also generate mitochondrial ROS (mROS), independent of NADPH oxidase activity[17] and, although less well characterized, mROS are thought to be important in neutrophil apoptosis[17] and NETosis[18]. Although predominantly active within the phagolysosome ROS may also diffuse into the cytosol and may cross the cell membrane into the extracellular environment where they may act as signalling molecules. Additionally, ROS have the capacity to alter the redox state of the cell which in turn has implications for signalling. Tyrosine phosphorylation is pivotal in cell proliferation, differentiation and in cell to cell communication. It is involved in transducing signals from diverse mediators including f-Met-Leu-Phe (fMLF), tumour necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Oxidants may alter the phosphorylation state of a protein either through inhibition of phosphatase activity or through induction of kinase activity[19] and therefore alter the activity of a wide range of signalling pathways.

Mechanisms of neutrophil cell death and implications for inflammation

An unusual facet of neutrophil biology is their propensity to undergo constitutive apoptosis, or programmed cell death. In both homeostasis and

the inflammatory response, the mechanism of neutrophil death is important. Apoptosis is a highly regulated and controlled process which results in cessation of both transcription and secretion of cytotoxins, effectively 'dismantling' the cytotoxic neutrophil machinery while maintaining the cytoplasmic membrane integrity and therefore avoiding release of toxins into the surrounding tissues. Apoptotic neutrophils are recognised by other immune cells, particularly macrophages which can take up (efferocytose) the dying cells and dispose of them in a safe fashion. Importantly, efferocytosis is a polarising stimulus for macrophages, stimulating a pro-resolution macrophage phenotype. Apoptosis can be triggered by two distinct pathways, the intrinsic and extrinsic pathways. The extrinsic pathway is activated by binding of cell surface death receptors such as the TNF-related apoptosis-inducing ligand receptors (TRAIL-R1 and TRAIL-R2) and the FAS receptor. Activation by their ligands, TRAIL and Fas Ligand respectively, leads to activation of the initiator caspases 8 and 10, facilitated by adaptor proteins found on the cytoplasmic domain of the death receptor. A cascade of caspase cleavage and activation ensues culminating in activation of the effector caspases 3 and 7 which trigger apoptosis[20]. The intrinsic pathway is, as the name suggests, activated by intracellular stress signals such as DNA damage or metabolic stress. In many cell types hypoxia is also an apoptotic signal, however, in neutrophils, hypoxia is a profound survival stimulus[21]. Defining hypoxia, both experimentally and in physiological conditions is discussed in more detail below. Stress signals result in activation of BCL-2 homology domain 3 (BH3)-only protein which in turn activates Bax and Bak activity,

resulting in mitochondrial outer membrane permeabilisation. This membrane permeabilisation leads to release of mitochondrial intermembrane space proteins such as SMAC (second mitochondria derived activator of caspases) and cytochrome *c* into the cytoplasm which ultimately activate caspase 9, triggering apoptosis through activation of the effector caspases 3 and 7. Anti-apoptotic proteins such as BCL-2 and MCL1 prevent mitochondrial membrane permeabilisation through interactions with BH3-only proteins and the pro-apoptosis Bax and Bak. MCL1 is the primary anti-apoptotic protein in neutrophils and, in comparison to the pro-apoptotic Bax, MCL1 has a relatively short half-life. This imbalance is thought to contribute to constitutive neutrophil apoptosis in the absence of survival stimuli[22]. Survival stimuli for neutrophils are diverse and include bacterial products such as LPS but also a number of cytokines, including GM-CSF, G-CSF, IL-1β, IFN-γ and TNF-α. Their downstream signalling converges on overlapping pathways including PI3K, ERK and NFκB to tip the balance of pro- versus anti-apoptotic factors and delay apoptosis.

Failure of neutrophil apoptosis can lead to persistence of neutrophilic inflammation and delayed neutrophil apoptosis has been suggested to contribute to both ARDS and sepsis. *Ex vivo* culture of blood neutrophils from patients with sepsis revealed an inverse correlation between rates of neutrophil apoptosis and sepsis severity and neutrophil apoptosis was lower still in those patients who developed sepsis related ARDS[23]. Soluble factors in the BAL fluid of patients with ARDS can prolong neutrophil survival *in vitro*[24] and neutrophils isolated from the blood of patients with ARDS show

reduced apoptosis compared to healthy controls following *ex vivo* culture[25]. Furthermore, there are lower rates of apoptosis in the peripheral blood neutrophils of patients with cystic fibrosis[26] and sputum neutrophils of patients with COPD[27].

Failure of neutrophil apoptosis leads not only to persistent neutrophilic inflammation but may also result in necrosis of neutrophils, another mechanism of cell death which, unlike apoptosis, results in loss of cytoplasmic membrane integrity and therefore release of cytotoxins into the surrounding tissues. This may also occur in secondary necrosis if the apoptotic neutrophils are not cleared by efferocytosis in a timely manner. Apoptosis is an active, ATP requiring process and so, energetic failure may contribute to neutrophil necrosis.

More recently a novel mechanism of neutrophil cell death, termed NETosis has been described. Neutrophil extracellular traps (NETs) are formed of chromatin, DNA, histones and granular proteins such as elastase or MPO and have bactericidal activity[28]. NETs were subsequently found to form during an active cell death process, dependent on ROS production and distinct from apoptosis and necrosis: NETosis[29]. Although first described *in vitro*, intravital microscopy has subsequently shown that NET formation and NETosis also occur *in vivo*[30]. The requirement for ROS is confirmed by the absence of NETs in patients with CGD[29] and the restoration of anti-fungal NET formation by restoration of NADPH oxidase function in these patients[31]. There is an alternative, NADPH oxidase independent NETosis pathway although this is less well defined. NETosis can be stimulated by microbes, antibodies and

chemical stimuli such as phorbol 12-myristate 13-acetate (PMA) and plays an important role in immunity through their capacity to kill large extracellular pathogens (such as *Aspergillus* species) and trapping pathogens to prevent disseminated infection[32]. Like many neutrophil antimicrobial functions, NETosis, if dysregulated, also has the capacity to cause inflammatory injury. This has been demonstrated in the lung in COPD[33] and cystic fibrosis[34].

Inflammation and Infection in the Lung

Gas exchange in the lung

The primary function of the lung is to allow gas exchange, and this is achieved through the pulmonary circulation. Deoxygenated blood moves from the right ventricle to the pulmonary arteries which branch to form pulmonary arterioles and then capillaries. The capillaries are in direct contact with the alveolus, the terminal point of the airways and it is at this point that gas exchange occurs by diffusion. This diffusion relies upon a number of important anatomical factors including the close apposition of the capillary basement membrane with the alveolar epithelial basement membrane, the vast surface area of the alveoli (approximately 70 m^2) and the narrow aperture of the pulmonary capillaries which ensures that red blood cells are close to the endothelial surface. The lungs also have a second circulatory supply from the left heart consisting of the bronchial arteries which supply oxygenated blood to the more proximal airways. There are some anastomoses between the two circulations but most blood returns to the left heart via the pulmonary veins and then into the systemic circulation. An important and unique aspect of the pulmonary circulation is ventilation-perfusion coupling: unlike most other tissues, in response to hypoxia the pulmonary circulation vasoconstricts, thus avoiding funnelling blood through poorly ventilated areas of lung where gas exchange cannot take place. This may occur in multiple respiratory pathologies where inflammation, infection or fibrosis may lead to thickening of the extracellular matrix and impaired diffusion and/or damage to the alveolar epithelial cells and impaired mucus clearance. Therefore, despite its role in oxygenating the

blood, areas of the lung may be subject to marked hypoxia in the diseased state. When extensive lung injury occurs, this failure of gas exchange may lead to systemic hypoxia, as well as localised hypoxia and this is often associated with significant morbidity and mortality.

Defining normoxia and hypoxia

In human physiology, when breathing room air (21% O_2) the normal arterial partial pressure of oxygen (pO_2) is approximately 10-12kPa. In the deoxygenated venous blood, the normal pO_2 is 4-5kPa. Measuring oxygen tension in living tissues is technically challenging, methods include imaging modalities such as positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). PET uses injected tracer compounds and indirectly measures oxygenation based on the redox status of cells. Its utility is limited by anatomical resolution[35]. MRS provides a higher level of resolution and, using perfluorocarbon probes, can measure absolute O_2 levels[36]. Other techniques which have been described include oxygen electrodes which rely on reduction of O_2 by a negatively charged noble metal electrode and, more recently, fibre-optic techniques. Fibre-optic measurement is advantageous as, unlike the electrodes, the probe does not consume the oxygen within the microenvironment. Much of the work in this area has focussed on hypoxia in the tumour microenvironment but some studies have attempted to define the 'normal' pO_2 of specific tissues and organs. The kidney, intestine and liver have been reported to have heterogenous oxygen levels. Measurement of renocortical oxygen levels in patients undergoing kidney transplant found a relatively high mean pO_2 of 9.5kPa[37] while intra-

operative measurement of intestinal oxygen levels found the pO2 to be 7-8kPa[38]. However, both the kidney and liver have also been found to have areas of much more marked hypoxia, particularly in the renal medulla. Parts of the skin are found to be profoundly hypoxia, with a pO_2 of <2kPa. A study of patients with lung tumours measured the pO2 in normal lung tissue at the time of tumour resection and this showed a highly variable degree of oxygenation (which the authors suggest may reflect incomplete lung deflation) but the median pO2 in normal lung was 5.6kPa[39]. These studies show that, even in the absence of inflammation, cells, particularly circulating leucocytes, may be exposed to a wide range of oxygen concentrations. True “normoxia” is therefore difficult to define and will vary from tissue to tissue and this poses an experimental challenge. In the literature and throughout this thesis, for *in vitro* experiments, normoxic culture is defined as 21% O_2, i.e. normal room air while *in vitro* hypoxia is defined as 1% O_2. Cells in culture may therefore be exposed to higher oxygen concentrations than would be expected in the tissue or circulation and so, while cell culture in these extremes of oxygen levels is an important tool in the investigation of hypoxic responses it is important to consider more physiological models in parallel. One such example which will be used in this thesis is the exposure of mice to hypoxia which, it is hoped, will provide a more physiological hypoxic challenge.

Neutrophils in the lung

In both infective and inflammatory disorders, the recruitment of immune cells to the airways occurs rapidly. In the context of infection, such as pneumonia, this is a critical step in containing and eradicating the infectious agent.

However, dysfunctional neutrophilic inflammation may be harmful and, if inappropriately activated, neutrophils have the capacity to cause severe damage to host tissues. In the lung, neutrophilic inflammation is implicated not only in acute diseases such as ARDS [40] but also in chronic disorders including COPD [41], cystic fibrosis [42] and bronchiectasis. The degree of neutrophilic infiltration in these diseases often correlates with disease severity and with lung damage. Furthermore, these chronic diseases are also characterised by recurrent and chronic infection, suggesting that the recruited neutrophils are simultaneously damaging and immunologically incompetent. There is therefore considerable interest in developing therapeutics which specifically target neutrophilic inflammation. This poses a significant challenge as damping down the harmful inflammation whilst preserving essential innate immunity represents a delicate balancing act.

Understanding the factors which influence the nature of the neutrophil response in the lung will be essential in targeting neutrophilic inflammation with the ultimate goal of resolving inflammation and restoring immune function.

Pathogenesis of acute respiratory distress syndrome (ARDS)

ARDS may occur in both pulmonary and extra-pulmonary diseases (such as burns, sepsis or pancreatitis). It is associated with a high mortality rate and, despite being first described in the 1970s, current therapeutic strategies revolve around supportive therapy with no specific licenced therapy available. This is reflected in the minimal improvement in outcomes over recent decades. Irrespective of the underlying cause, ARDS is characterised by protein-rich oedema in the alveoli and lung interstitium, attributable to increased pulmonary

vascular permeability[43]. The primary cause of the vascular injury is neutrophil mediated damage[44]. There is evidence that additional alveolar epithelial injury is required, in parallel with the vascular injury, for the development of ARDS. Epithelial injury may occur in response to a chemical stimulus, (for example acid from aspirated gastric contents or inhalation of toxins) or a pressure injury, particularly in mechanically ventilated patients[45,46]. However, like the vascular injury, epithelial damage is most commonly caused by the release of factors from infiltrating immune cells, particularly neutrophils. The neutrophil is therefore an attractive therapeutic target in this disease because it is essential in the pathogenesis and perpetuation of the inflammatory response. However, in order to develop such a therapy, a more comprehensive understanding of how and why the dysfunctional neutrophilic inflammation develops is required.

Microenvironmental regulation of inflammation

The vital role of the microenvironment in regulating inflammatory responses is increasingly recognised. In addition to the DAMP and PAMP signals described above, multiple other environmental stimuli including the cytokine environment, nutrient availability and oxygen tension play a part in shaping the inflammatory response.

Cytokines and neutrophil priming

Neutrophils are exquisitely sensitive to the cytokine environment and possess a wide range of cytokine receptors. Cytokines acting upon neutrophils can be divided into different classes with different structures, receptors and signalling pathways.

Type I cytokines consist of 4 α-helices and include IL-4, IL-6, granulocyte colony-stimulating factor (G-CSF) and GM-CSF. Type II cytokines contain 6 α-helices and include Interferon (IFN)-α, -β and -γ and IL-10. Both Type I and type II cytokine signalling is mediated by JAK-STAT pathways. The IL-1 super family includes the three known forms of IL-1 (IL-1α, IL-1β and IL-1Ra) and IL-18. IL-1β is a critical regulator of the inflammatory response and is predominantly produced by macrophages but may also be produced by epithelial cells and fibroblasts. In neutrophils its main effect is to prolong survival. TNF-α is a key inflammatory cytokine. It is produced in large quantities by activated macrophages and can act upon both macrophages and neutrophils. It is important in priming and activating neutrophils.

Neutrophil priming describes the phenomenon whereby exposure to a 'priming' stimulus results in an amplified response from the neutrophil following

subsequent activation. Neither the priming stimulus nor the activating stimulus in isolation is able to unleash the full antimicrobial potential of the neutrophil but together they induce a potent effector cell function. Priming agents include pathogen derived factors such as LPS and the influenza A virus and 'self' inflammatory mediators such as TNF-α[47], IL-8 and GM-CSF[48]. These priming stimuli may be generated by other immune cells or by endothelial cells. The consequences of priming are manifold: classically priming is measured by enhanced ROS production and increased phagocytic activity, however, primed neutrophils may also alter the make-up of their surface receptors, thus changing their capacity to respond to subsequent cytokine signals. Primed neutrophils also show enhanced degranulation and survival. The physiological importance of priming is clear: a fully activated neutrophil is potentially highly toxic to host tissue, but neutrophils must be activated quickly to be effective when required to fight infection. The intermediate primed stage avoids having high numbers of active neutrophils in the circulation but means that in response to a danger stimulus, there is a rapidly available population of potent effector cells.

Interestingly, priming may be reversible in response to certain stimuli, including platelet activating factor (PAF). This 'depriming' is also a safety mechanism – if after a period of priming, the neutrophil is not fully activated it can 'stand down' again, retaining the capacity to be reprimed and fully activated at a later stage[49]. Recent studies in acute lung injury suggest that a failure of this depriming may contribute to the neutrophil mediated cell damage seen in this condition[50].

Hypoxic regulation of inflammation and immunity

Multiple links exist between hypoxia signalling pathways and inflammatory processes. Inflammation may lead to local or systemic hypoxia, for example in an abscess where lack of blood supply results in profound local hypoxia or in lung inflammation (such as ARDS) where impaired gas exchange can lead to systemic hypoxia. Additionally, an influx of inflammatory cells may lead to hypoxia as the demand for oxygen outstrips supply, known as inflammatory hypoxia[51]. The links between hypoxic signalling and inflammation are bidirectional: hypoxia may also exacerbate inflammation through activation of inflammatory pathways and through effects on immune cell fate and function.

Cellular responses to hypoxia are essential for life and are now recognised to be dysregulated in a wide range of disease processes. The key players in these pathways are a family of transcription factors, the Hypoxia Inducible Factors (HIFs) and their regulatory proteins, the prolyl hydroxylase domain enzymes (PHDs) and Factor Inhibiting HIF (FIH). The active HIF transcriptional complex is a heterodimer of the oxygen sensitive HIFα subunit and the constitutively expressed HIFβ subunit (also known as the aryl hydrocarbon receptor nuclear translocator, ARNT)[52]. This complex activates transcription through binding to Hypoxia Response Elements (HREs) present on a broad range of genes (Figure 1.1, adapted from [53]), thus regulating the hypoxic response.

Figure 1.1

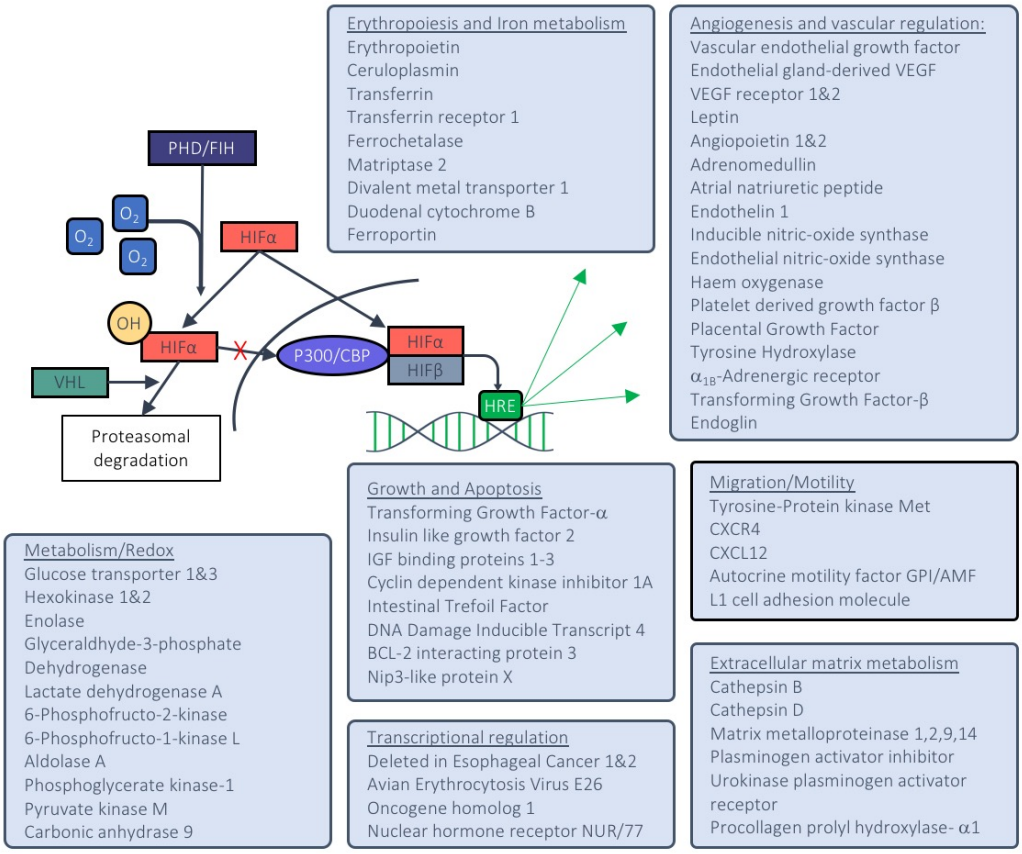


Figure 1.1 The hypoxic response is regulated by HIFs and their inhibitory hydroxylases The Hypoxia Response Is Regulated by Hypoxia-Inducible Factors (HIFs) and Their Inhibitory Hydroxylases. The active form of HIF comprises an α and a β subunit. The prolyl hydroxylase domain enzymes (PHDs), and an asparaginyl hydroxylase, factor inhibiting HIF (FIH), act as the cellular oxygen sensors: these require oxygen for hydroxylation of the HIFα subunit. Prolyl hydroxylation targets HIFα for proteasomal degradation by the von Hippel–Lindau (VHL) protein and asparaginyl hydroxylation prevents binding of the transcriptional co-factor p300/CBP, preventing HIF-mediated transcription under oxygen-replete conditions.

The hypoxia-sensing pathways are highly conserved, and the prolyl hydroxylase domain enzymes may also be termed EGLNs due to their early identification as homologues of the C. Elegans oxygen sensor EGL-nine[54]. In this nomenclature PHD1, 2 and 3 are known as EGLN 2,1 and 3 respectively. The PHDs are part of a family of 2-oxoglutarate (2OG) and Fe(II)-dependent hydroxylases. Protein hydroxylation is increasingly recognised as an important post-translation modification which may directly alter protein structure, provide a stepping stone for further modifications or regulate protein-protein interactions[55]. The first described 2-OG dependent hydroxylases were those which catalysed proline hydroxylation in procollagen, regulating the stability of the collagen triple helix[56]. The PHDs hydroxylate prolyl residues within the Oxygen Dependent Degradation Domain (ODDD) of the HIFα subunit[57]. This hydroxylated form of HIFα binds the Von Hippel Lindau protein which has E3 ubiquitin ligase activity and targets the HIFα subunit for proteasomal degradation[58]. Prolyl hydroxylation by PHDs requires molecular oxygen and 2-OG as substrates and therefore provides the oxygen-sensing element of the pathway – in the absence of oxygen, HIFα cannot be hydroxylated and, instead of being degraded, accumulates and translocates to the nucleus where it binds the HIFβ subunit and activates transcription. The requirement for 2-OG also links hypoxic signalling to metabolic processes and this will be discussed in more detail below. Factor Inhibiting HIF (FIH) is another 2-OG-dependent hydroxylase which also acts as a cellular oxygen sensor through a distinct mechanism[59]. FIH hydroxylates asparagine residue 803 in the HIFα subunit which, instead of targeting it for degradation

prevents HIFα C-terminal transactivation domain (CAD) interactions with the coactivator P300, thus preventing HIF mediated transcriptional responses[60]. Over the last half century, hydroxylation by 2-OG dependent enzymes has been found to regulate gene expression at multiple levels. Hydroxylation by the 2-OG dependent Jumonji C domain (JMJD) containing proteins regulates histone methylation status, an important factor in transcriptional activity. The JMJD containing protein JMJD6 has also been implicated in the regulation of RNA splicing[55]. Additionally, the ten-eleven translocation (TET) group of 2-OG oxygenases alter DNA methylation status by converting 5-methyl cytosine residues to 5-hydroxymethyl cytosine which is, through base excision repair, replaced with an unmethylated cytosine[61]. The suppression of TET activity during hypoxia in the tumour microenvironment was associated with hypermethylation of predominantly enhancer and promotor regions[62]. Hypermethylation of tumour suppressor gene promotors results in reduced expression of these proteins and has been hypothesised to drive tumorigenesis. Thus, through the action of this family of 2-OG dependent hydroxylases, oxygen availability regulates gene expression at multiple levels, shaping cellular identity and behaviour.

Isoform specific roles for HIFs and PHDs

An important consideration in understanding cellular responses to hypoxia is the added capacity to 'fine tune' the hypoxic response which is afforded by the existence of multiple isoforms of both HIFα and PHD.

To date three isoforms of HIFα have been described with HIF-1α and HIF-2α being the best characterised and most ubiquitously expressed. HIF-3α is the

most structurally distinct isoform and is present in multiple splice variants which have complex and variable roles[63]. While HIF-3α may regulate transcription of some hypoxia inducible genes, it also has a role in regulating HIF-1α and HIF-2α activity. Despite similarities in structure and function of the HIF-1α and HIF-2α isoforms, there are key differences in their transcriptional repertoire which provide an additional level of complexity in the hypoxic transcriptional response. This differentiation is multifactorial, relating to variations in tissue expression, temporal regulation and differences in gene specificity. Interestingly, this specificity is driven not by the DNA binding domain but by the N-terminal transactivation domain[64]. The balance of HIF-1α versus HIF-2α has important implications for inflammation outcome.

The three PHD isoforms found in mammals are thought to have arisen through gene duplication early in vertebrate evolution[65] and are represented by a single isoform in *Caenorhabditis elegans*, Egl9. While the Egl9 gene is essential for regulation of HIF in C elegans, all three mammalian isoforms have HIF hydroxylation activity[54]. However, an increasing body of evidence suggests that the PHD isoforms are non-redundant in mammalian cells and the specific function of each PHD isoform is the subject of extensive and ongoing research. Pharmacological inhibition of specific PHDs is not yet possible, although current compounds may have a degree of selectivity. However, generation of cell-specific, isoform specific, knockout animals and the use of other techniques such as SiRNA have enabled researchers to begin to elucidate the function of specific PHDs. All three PHDs are widely expressed in cells of the innate and adaptive immune system. PHD2 is generally

considered to be the dominant isoform in the regulation of HIFα under physiological oxygen tensions[66] while PHD3 is often upregulated in hypoxic conditions. It is also important to consider that the PHDs have been postulated to have HIF independent activity in the context of inflammation and malignancy. NF-κB is a transcription factor which, like HIF, regulates a diverse group of genes including cytokines, immune receptors and stress response genes[67]. In some contexts NF-κB activity is linked to hypoxic signalling in a HIF dependent fashion[68,69]. However, there is also evidence that PHD activity may directly regulate NF-κB, impacting inflammatory and malignant outcomes[70-72]. FIH has also been found to regulate NF-κB activity and knockdown of FIH and PHD1 had an additive effect in reducing NF-κB activity[73]. This cross talk between inflammation and hypoxia has important implications in infection, sterile inflammation, ischaemic injury and within the tumour microenvironment.

HIFs/PHDs in neutrophil Biology

As the first responders of the innate immune response, neutrophils are required to function in a hostile microenvironment, and this often incudes exposure to marked hypoxia. Neutrophils express all of the oxygen sensing machinery described above (although there is little data in the current literature regarding the role of FIH in neutrophils). In the context of an acute inflammatory response, neutrophils demonstrate distinct temporal expression profiles of the HIFα isoforms [74], with early upregulation of HIF-1α in an acute lung injury model (6 hours post-stimulus) and delayed HIF-2α upregulation, peaking as the HIF-1α levels fall at 24 hours. Importantly, loss of HIF-2α in

myeloid cells results in enhanced resolution of neutrophilic inflammation (associated with a pro-apoptotic neutrophil phenotype), consistent with HIF-2α being the dominant isoform in the latter stages of the neutrophilic inflammatory response. Additionally, expression of HIF isoforms varies with neutrophil type with differential expression noted in bone marrow, blood and inflammatory tissue neutrophils. HIF-1α has been shown to be essential for a range of myeloid cell functions[75], including delayed neutrophil apoptosis in hypoxia[69]. In contrast to this, loss of HIF-2α in neutrophils does not result in loss of hypoxic survival[74]. These data highlight the importance of environmental context and timing in determining the role of individual HIF isoforms in the inflammatory response.

Given the critical role of HIF in neutrophil survival and function, it is of little surprise that manipulation of PHD activity has profound consequences for the neutrophil and PHD2 and PHD3 have both been shown to regulate neutrophilic inflammation. Genetic ablation of PHD2 in myeloid cells or pharmacological inhibition of PHD2 activity resulted in exaggerated inflammation in response to the pathogen *Streptococcus Pneumoniae*. This was not due to a failure of pathogen clearance but rather an over-exuberant neutrophil response, also noted in response to sterile, LPS induced inflammation. This resulted in increased migration, enhanced neutrophil survival and upregulation of glycolysis with consequent increased energy charge[76]. Importantly, this response was associated with increased stability of HIF-1α. In contrast to these findings, investigation of the role of PHD3 in neutrophilic inflammation identified an unexpected phenotype[77]. Both hypoxia and inflammatory

stimuli were found to increase levels of PHD3 in neutrophils (consistent with findings in other cell types[66]). Interestingly, despite preserved HIF transcriptional activity (as measured by synthesis of the HIF target genes GAPDH and Glut1), loss of PHD3 led to a failure of neutrophil hypoxic survival. This had important consequences *in vivo* with improved resolution of LPS induced acute lung injury and reduced inflammation in a mouse model of colitis. Taken together, these data highlight the isoform specific actions of PHDs. Studies of macrophage function in the context of PHD3 loss highlight the cell specific functions of these enzymes. In a model of LPS-induced peritoneal inflammation which is predominantly macrophage mediated, loss of PHD3 results in an overwhelming innate immune response which was lethal[78], in contrast to the neutrophil phenotype described above. These data demonstrate that hypoxia, and hypoxic signalling pathways, may be either pro- or anti-inflammatory and the context of the inflammatory insult is of vital importance in determining the outcome. The impact of hypoxia on the infiltrating immune cell population must also be balanced with the effect of hypoxia on the tissue in question. As discussed above, inflammation in the lung, and adaptive ventilation-perfusion matching may lead to areas of hypoxia within the lung and HIF activity has also been shown to have both beneficial and detrimental effects in the lung tissue.

HIFs/PHDs in the Lung

The lung is exposed to an enormous range of foreign but innocuous material and so must have robust immune tolerance mechanisms. However, it is also susceptible to both sterile inflammation and infection with pathogens including

viruses, bacteria and fungi. HIF-1α is stabilised in alveolar epithelial cells in response to inflammatory stimuli[79]. Resolution and repair in the alveolus is dependent upon proliferation and trans-differentiation of alveolar cells and HIF-1α dependent VEGF expression was found to be critical for this response[79]. HIF-1α was also stabilised in response to stretch injury, consistent with mechanical ventilation. Interestingly, this normoxic induction of HIF was also found to be protective in acute lung injury (ALI) models[80]. HIFα stabilisation was dependent on induction of succinate dehydrogenase and consequent inhibition of PHD activity (discussed in detail below). Alveolar epithelial cell specific HIF-1α deletion is associated with increased morbidity and mortality in stretch induced ALI. In contrast to these findings, Xi et al[81] found that, in the context of influenza induced alveolar epithelial cell loss, HIF-1α activation may be associated with dysfunctional alveolar remodelling. The authors found that hypoxia, dependent on HIF-1α activity, was key in determining epithelial cell fate and that mice lacking epithelial cell HIF-1α recovered more quickly with improved expansion of the type II alveolar cell population and restoration of normal epithelium. In addition to these findings focussing on acute lung injury, both HIF-1α and HIF-2α in the lung epithelium have been shown to play a role in chronic, cobalt induced, lung inflammation[82,83]1. Loss of HIF-1α was associated with a switch from type 1 inflammation (characterised by cytokines such as TNF-α and IL-2) to type 2 (allergic-type) inflammation with fewer neutrophils but increased eosinophilic inflammation and increased Th2 cytokines such as IL-4[82]. HIF-2α deletion was similarly associated with enhanced and prolonged eosinophilic

infiltration[83]. Interestingly, the time frame of this eosinophilic inflammation varied depending on which HIFα isoform was targeted, consistent with other studies showing that the predominant isoform varies over the time course of an inflammatory response[74], with HIF-2α appearing to predominate in the latter, reparative stages of inflammation in the lung.

The impact of hypoxia on an inflammatory response in the lung is therefore difficult to predict and likely to be highly context specific.

Metabolism and metabolites in inflammation regulation

Neutrophils are unusual in that, even in the quiescent state, they generate the vast majority of their ATP through glycolysis, irrespective of oxygen availability. One major advantage of this is their capacity to generate energy rapidly in hypoxic environments. However, metabolic intermediaries also have functions out-with ATP generation, including in modulation of immune responses.

Metabolic Intermediaries in Immunity

The field of immunometabolism is rapidly expanding as novel roles for metabolic intermediaries are discovered. There are also close links between metabolic signalling pathways and the HIF/PHD hypoxic signalling pathways described above (Figure 1.2). The TCA cycle intermediary 2-oxoglutarate is an essential substrate for PHD and FIH activity. However, other TCA cycle intermediaries including fumarate[84], succinate[85] and citrate[86] can inhibit PHD activity. Succinate is also produced during the hydroxylation of HIF by PHDs, providing a potential feedback mechanism.

Figure 1.2

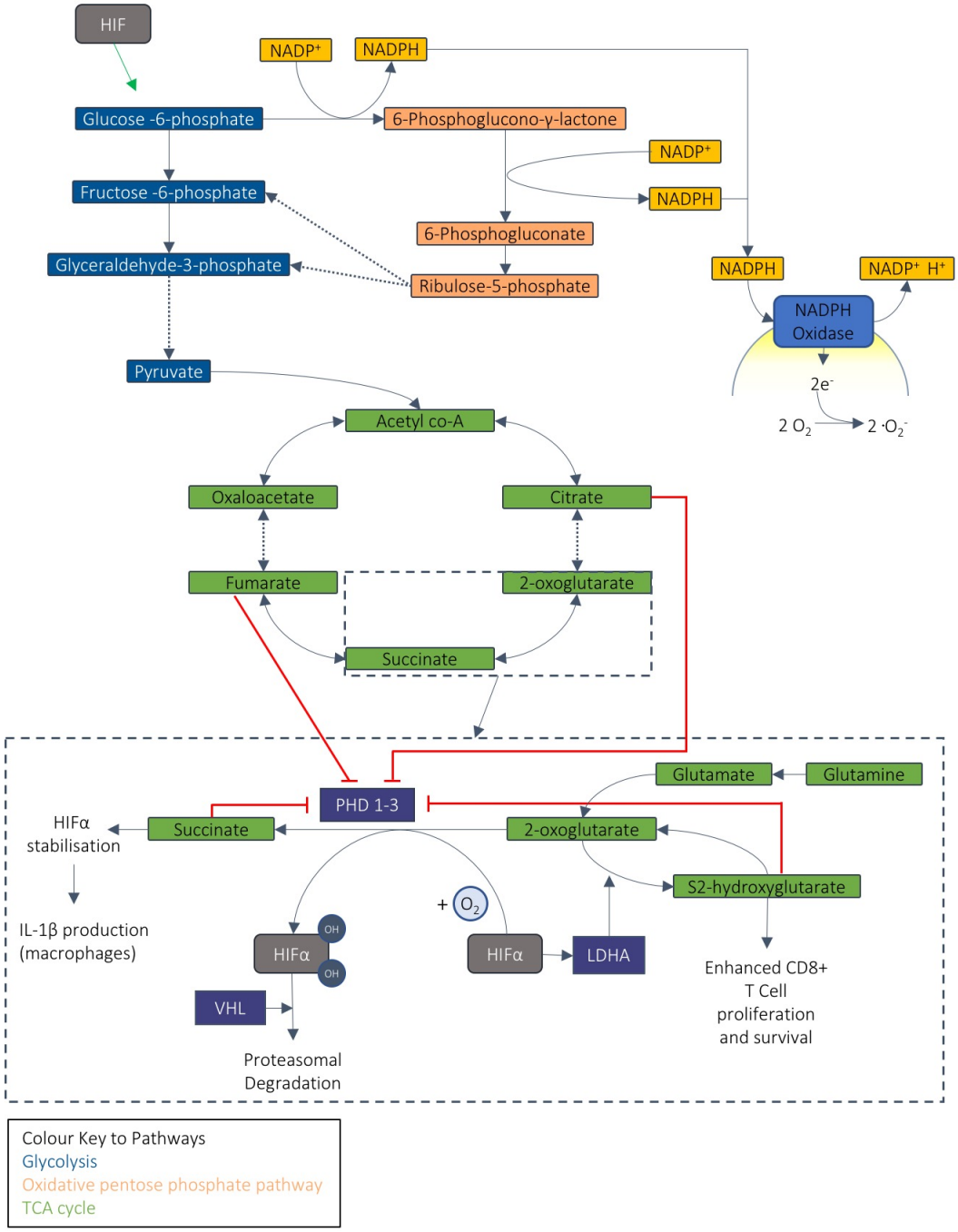


Figure 1.2 Multiple links exist between metabolic and hypoxic signalling pathways
HIF activation leads to enhanced glycolysis through HIF mediated transcription of glycolytic enzymes and glucose transporters. HIF activity is, in turn, regulated by a number of TCA cycle intermediaries through their effects on PHDs. Metabolic factors may also regulate inflammation, for example through ROS production, polarisation of monocytes and regulation of CD8+ T cell functions.

Metabolic adaptations are also essential in the polarisation of immune cells, particularly macrophages and T cells and HIF mediated transcription is closely linked to these metabolic responses. Macrophage activation is associated with a metabolic switch from oxidative phosphorylation (OxPhos) to glycolysis[87]. This phenotype is favoured by HIF-1α mediated transcription of glycolytic genes and glucose transporters and occurs in both hypoxia and in response to inflammatory stimuli such as LPS. HIF-2α may be dispensable for regulation of some key glycolytic genes[88]. Tannahill et al[89] identified succinate as a HIF-1α dependent inflammatory signal in macrophages: succinate production by macrophages is required for HIF-1α stabilisation which is, in turn, required for production of the cytokine IL-1β. In a study which has fascinating parallels with this work in macrophages, Tyrakis et al[90] describe the critical role played by HIFα dependent accumulation of the metabolite S-2-Hydroxyglutarate (S-2HG) in memory $CD8^+$ T cell development. The authors demonstrate that S-2HG can enhance proliferation and persistence of memory $CD8^+$ T cells through altered epigenetics with consequences for T cell anti-tumour activity. In both of these papers, glutamine is an important source of the succinate or S2HG through anaplerosis.

It is not only metabolic intermediaries and enzymes which can influence immunity but also the metabolic sensing machinery of the cell and this sensing machinery is also closely associated with the hypoxic signalling pathway.

mTOR and AMPK sense the metabolic microenvironment

The serine/threonine kinase mammalian (or mechanistic) target of rapamycin (mTOR) is a critical regulator of cellular metabolism. mTOR forms two different

kinase complexes with discrete upstream regulators and downstream targets. This selectivity is determined by the complex specific components. Both mTORC1 and mTORC2 contain mTOR (the catalytic component) and mLST8 while RAPTOR and RICTOR are specific to mTORC1 and 2 respectively. RAPTOR (regulatory protein associated with mTOR) binds TOR signalling motifs on mTOR substrates, thus recruiting them to the mTORC1 complex and also plays a role in subcellular localisation of the complex. RICTOR performs a similar role within the mTORC2 complex[91]. The role of mLST8 (also known as GβL) in this context is complex and varies with the stage of embryonic development and between mTORC1 and 2. In cell culture systems, acute knock down of mLST8 by SiRNA, demonstrated that its expression is necessary for mTOR mediated phosphorylation of the downstream kinase S6K and also influences the nutrient sensitive interaction between mTOR and RAPTOR[92] and thus was essential for mTORC1 signaling. However, embryonic ablation of mLST8 showed that, in development, mTORC1 signalling does not require mLST8 but the mLST8 deficient embryo was a phenocopy of the RICTOR deficient embryos. mLST8 was required for mTOR/RICTOR interactions and therefore mTORC2 signalling[93]. Both mTORC1 and 2 also contain additional regulatory subunits.

Figure 1.3

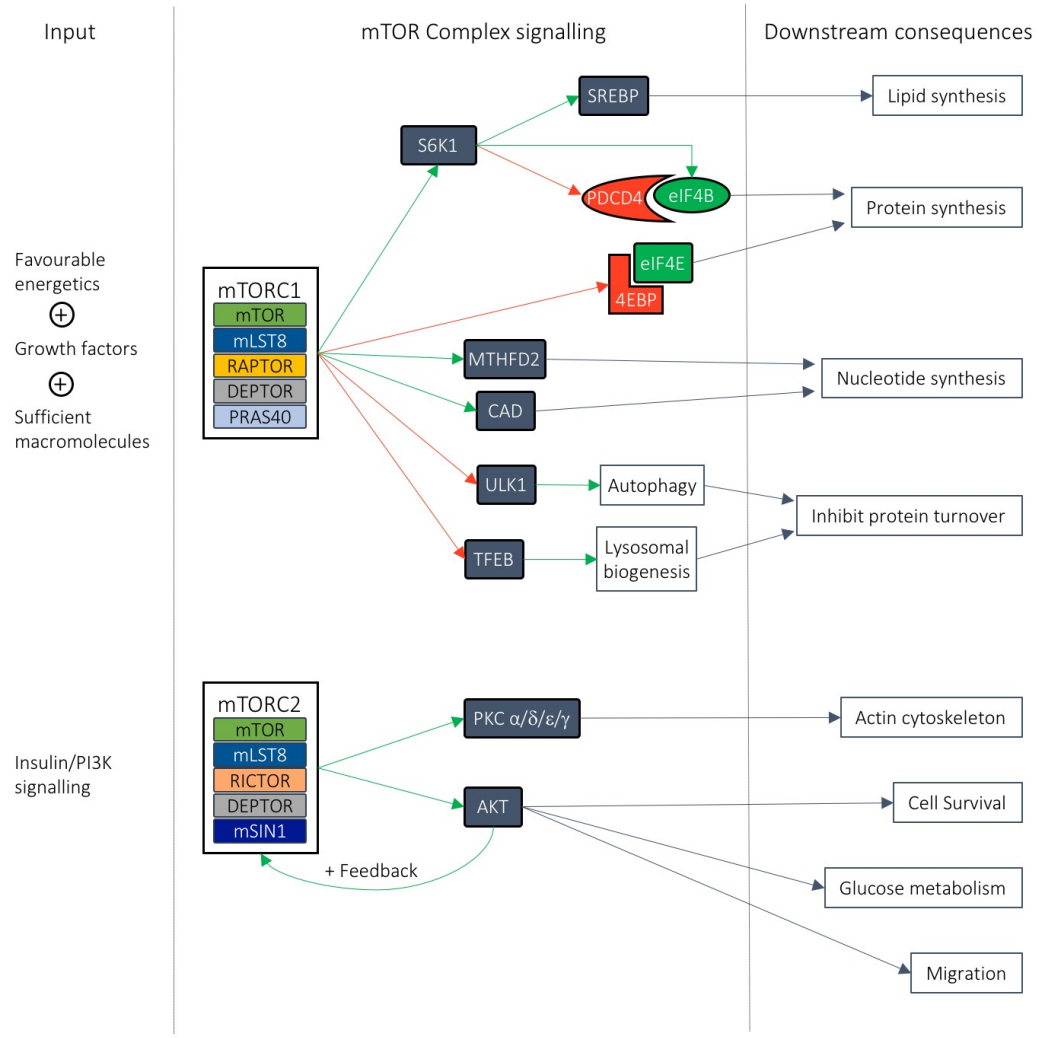


Figure 1.3 mTOR complexes regulate metabolism, survival and growth
mTORC1 and mTORC2 contain some common and some specific proteins, as illustrated. These include both activating and inhibiting cofactors and also molecules which target the complex to specific intracellular locations (RAPTOR and RICTOR). mTORC1&2 are activated by distinct upstream signals and, through phosphorylation of multiple downstream targets, facilitate metabolic and biosynthetic adaptations to environmental stimuli.

Active mTORC1 drives anabolic processes, particularly protein synthesis in conditions which are suitable for growth, i.e. in response to endocrine growth signals, favourable cellular energetics and adequate macromolecular building blocks such as amino acids (Figure 1.3). Two key downstream targets of mTORC1 are p70S6 Kinase 1(S6K1) and eIF4E binding protein (4EBP) which act to regulate translation through regulation of eukaryotic initiation factors (eIFs). S6K acts directly through phosphorylation and activation of eIF4B and indirectly via phosphorylation, and therefore enhanced degradation of the protein programmed cell death 4 (PDCD4) which would otherwise inhibit eIF4B. mTORC1 mediated phosphorylation of 4EBP results in its dissociation from eIF4E, allowing formation of the eIF4F complex and therefore promoting translation. In addition to regulation of protein synthesis, mTORC1 also regulates lipid synthesis via activation of the sterol responsive element binding protein (SREBP) and both purine and pyrimidine synthesis via activation of the mitochondrial tetrahydrofolate cycle[94] and carbamoyl-phosphate synthetase (CAD) [95,96] respectively. mTORC1 also plays an important part in regulating protein catabolism and the related process of autophagy. mTORC1 phosphorylates the essential component of the autophagosome ULK1 which precludes its activation by the AMP activated protein kinase (AMPK). Additionally, mTORC1 phosphorylates the transcription factor TFEB which results in its retention within the cytoplasm, preventing TFEB mediated lysosomal biogenesis[97]. The lysosome is the primary site of intracellular protein catabolism, and is essential in autophagy and so, through this mechanism, mTORC1 acts to further suppress protein breakdown. Finally,

mTORC1 has been found to regulate glucose metabolism, inducing a switch from oxidative phosphorylation to glycolysis, at least in part through upregulation of HIF-1α and therefore the HIF target genes involved in glycolysis[98].

In contrast, mTORC2 activity requires release from its negative regulatory subunit mSIN1. This is achieved through PtdIns(3,4,5)P_3 (PIP3) binding the pleckstrin homology (PH) domain of mSIN1p[99], halting its inhibition of mTOR kinase activity and occurs downstream of insulin/PI3kinase signalling pathways. The major downstream target of mTORC2 is AKT which in turn promotes cell survival and growth. AKT may also phosphorylate mSIN1, promoting mTORC2 activity in a positive feedback loop. mTORC2 also phosphorylates and activates protein kinase C (PKC) family members which are involved in cytoskeletal rearrangement.

mTOR is thus a master regulator of cellular metabolic processes, able to respond to diverse stimuli and orchestrate a comprehensive response to the metabolic environment. An important parallel signalling pathway in this role is via AMP activated protein kinase (AMPK). In contrast to mTOR, AMPK is activated in response to metabolic stress, specifically by rising AMP:ATP ratios. The downstream action of AMPK is often the converse of mTOR activity. For example, AMPK switches on catabolic processes such as glucose uptake, fatty acid oxidation and autophagy and inhibits synthesis of proteins, fatty acids and glycogen[100]. The balance of mTOR and AMPK activity therefore has profound consequences for cell growth and survival and will be defined by the metabolic environment. Given the equally important role of

hypoxia in regulating these processes, it is perhaps unsurprising that there is considerable crosstalk between mTOR, AMPK and hypoxic signalling pathways. mTOR activity is supressed by hypoxia due to upregulation of the HIF target gene REDD1 and its subsequent activation of the mTOR inhibiting tumour suppressor complex (TSC) [101]. Hypoxia has been found to activate AMPK independently of HIF-1α[102] but chemical stabilisation of HIF has also been reported to increase phosphorylated (active) AMPK[103]. This hypoxia induced AMPK activity serves to further suppress mTOR activity in hypoxia due to both AMPK mediated TSC2 activation and through direct AMPK phosphorylation of RAPTOR[104].

mTOR and inflammation

There are contradictory reports on the regulation of inflammation by mTOR. While mTOR inhibitors, typified by Rapamycin, have been used as immunosuppressive therapies, particularly in organ transplant, mTOR inhibition has also been reported to have pro-inflammatory effects[105,106]. As with manipulation of HIF signalling, the cell type, microenvironmental context and timing of mTOR targeting are all likely to determine the inflammatory outcome. Relatively little is known about the role of mTOR in regulating neutrophilic inflammation. Consistent with its activation of AKT and PKC, mTORC2 regulates neutrophil migration through alterations in the actin cytoskeleton[107]. One study found that mTORC1 mediated HIF-1α was required for NET formation in neutrophils[108] however, another study found that mTOR inhibition with rapamycin led to accelerated NET formation[109]. Importantly, the stimulus for NET formation varied between these studies, and

this may have contributed to the contradictory results. mTOR has also been implicated in neutrophil cytokine generation with rapamycin treatment *in vitro* leading to reduced TNF-α and IL-6 production[110]. Lorne and colleagues also found that pre-treatment with intra-peritoneal rapamycin reduced the severity of LPS-induced lung injury although, whether this was due to a specific effect on neutrophils is not clear.

The potential role of mTOR in neutrophils is particularly interesting for three reasons. Firstly, as terminally differentiated cells, neutrophils do not proliferate and so their requirement for the building blocks of cell growth will be lower than those of proliferating cells such as T lymphocytes or tumour cells, thus the anabolic effects of mTORC1 activation may have different consequences in neutrophils. Secondly, neutrophils undergo constitutive apoptosis but, unusually, this is delayed in hypoxia and so it is possible that hypoxic inhibition of mTOR in neutrophils may alter cell survival. Finally, neutrophils are primarily glycolytic at baseline and so there is no “glycolytic switch” in response to metabolic or hypoxic stress (although, glycolysis can be further upregulated in activated neutrophils) so the effect of glucose deprivation on both mTOR and AMPK activity in neutrophils may also differ from that seen in other cell types.

Summary

The literature discussed above demonstrates the vital role played by neutrophils in innate immunity but also their capacity to contribute to pathology, particularly in the lung. This double-edged sword represents a significant challenge in the design of therapies which target neutrophil mediated disease. In order to address this unmet clinical need, a more comprehensive understanding of the pathways and processes which regulate neutrophil function during the inflammatory response is required. There is an increasing body of evidence which points to the microenvironment as a critical regulator of inflammatory responses. In the lung this will include variations in oxygen and nutrient availability as well as diverse cytokine signals. Inflammation, hypoxia and metabolism are inextricably linked with multiple points of crosstalk identified in their respective signalling pathways. How these factors regulate neutrophil function is of interest, in part due to specific aspects of neutrophil biology: mature neutrophils are non-proliferative and terminally differentiated; they exhibit high basal rates of glycolysis with very limited aerobic respiration and finally, they undergo constitutive apoptosis, which is reduced by hypoxia.

Aims

The broad aim of this thesis is to determine the mechanisms which govern hypoxic regulation of neutrophil function. More specifically, it will address the following questions:

- How does hypoxia regulate neutrophilic inflammation *in vivo* and what are the consequences of this regulation?
- What defines the proteome of inflammatory lung neutrophils and how is this regulated by hypoxia?
- How does the microenvironment of the hypoxic lung contribute to neutrophilic inflammation in this context?
- How do metabolic signalling pathways and hypoxic signalling pathways interact to allow neutrophils to sense and respond to microenvironmental stresses?

Chapter 2: Methods

Isolation and culture of human peripheral blood neutrophils

Human peripheral blood neutrophils were isolated by dextran sedimentation and discontinuous percoll gradient. Peripheral venous blood was taken from healthy volunteers with written informed consent as approved by the Centre for Inflammation Research Blood Resource Management Committee (AMREC 15-HV-013). Blood was drawn using a 21-gauge (21G) butterfly needle. 36ml of whole blood was transferred into a 50ml polystyrene falcon tube containing 4ml of 3.8% Sodium Citrate and mixed by gentle inversion of the tube. The anticoagulated blood was centrifuged at 350G for 20 minutes (acceleration 5, deceleration 5). The platelet rich plasma layer was carefully removed. 6ml of 6% dextran was added to the remaining cell-rich component and topped up to 50ml with pre-warmed 0.9% sodium chloride (NaCl). This was incubated at 37°C for 30 minutes to allow dextran sedimentation of the erythrocytes. The leucocyte rich top layer was then carefully removed and topped up to 50ml with 0.9% NaCl prior to centrifugation at 350G for 6 minutes to pellet the leucocytes. A discontinuous percoll gradient was then used to separate granulocytes from the whole leucocyte population. A stock solution of 90% percoll (GE Healthcare) was made using 10% vol/vol of 10X PBS. 73%, 61% and 49% percoll were made by dilution of the 90% stock with 1X PBS as detailed in Appendix 1. Gradients were made in 15ml polystyrene falcon tubes. 3ml of 73% percoll was pipetted into the bottom of the tube and 3ml of 61% percoll was gently layered on top. The leucocyte pellet was then resuspended in 3ml of the 49% percoll and carefully layered onto the 61% layer. The percoll

gradient was centrifuged at 720G for 20 minutes (acceleration 1, deceleration 0). The peripheral blood mononuclear cell layer was removed from the interface between the 49% and 61% layers. The granulocyte layer (at the 73%/61% interface) was removed carefully using a sterile pastette. The cells were then washed twice with sterile 1X PBS prior to haemocytometer counts and downstream use. Purity was assessed by morphology on cytocentrifuge slides.

Cytocentrifuge slide preparation

Cytocentrifuge slides (Cytospins) allow analysis of cellular morphology and were used throughout this thesis. The cytospin equipment consists of a glass slide, a filter paper and a cytospin chamber with a funnel for cells, held together with a slide holder. 100μl-200μl of cell suspension ($1x10^5$-$5x10^5$ cells) was pipetted into the funnel and the cytospin was centrifuged at 300 revolutions per minute (rpm) for 3 minutes in a CytoSpin3 cytocentrifuge (Thermo Scientific). Slides were fixed with 100% methanol and stained using the Shandon Kwik-Diff staining system (Thermo Scientific): Slides were first stained with Eosin and then with Methylene Blue to delineate nuclear structures. Slides were mounted using DPX moutant (Sigma-Aldrich) and a glass cover slide.

Mouse models

Mice

C57BL/6JOlA mice were purchased from Envigo, UK. Male mice aged 8-10 weeks were used in all experiments.

PHD1 whole animal knockout animals and $PHD1^{fl/fl}$ mice were a gift from Peter Carmeliet (VIB Vesalius Research Centre, KU Leuven, Belgium). $PHD1^{fl/fl}$ animals were crossed with MRP8-Cre-ires/GFP animals purchased from the Jackson Laboratory (stock number 021614) to generate neutrophil specific PHD1 deficient mice[111] (Figure 2.1) which will be referred to as follows: $PHD1^{fl/fl}MRP8Cre^{+/-}$ (knockout, KO) or $PHD1^{fl/fl}MRP8Cre^{-/-}$ (wild-type, WT). KO mice were healthy and born at normal mendelian frequency. Animals were age and sex matched for experiments and used at 2-3 months of age.

All mouse experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 with local ethics approval.

Figure 2.1

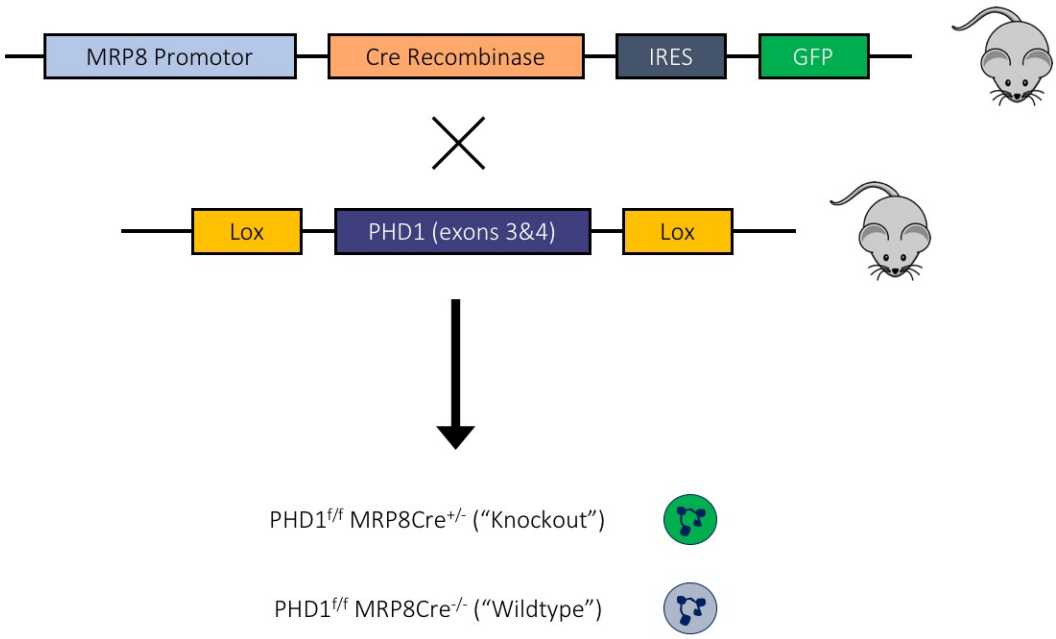


Figure 2.1: Generation of the PHD1$^{fl/fl}$ MRP8Cre$^{+/-}$ mouse line
Mice which were heterozygous for the MRP8 driven Cre recombinase were crossed with homozygous PHD1 floxed mice to generate neutrophil specific PHD1 deficient mice (Cre^{+}) and wild-type litter mate controls (Cre^{-}).

In Vivo mouse models

Exposure to Hypoxia

Mice were exposed to hypoxia (10% inspired O_2) in an InVivo Hypoxic Cabinet System (Coy Labs, USA). This sealed system allows animals to be housed in 10% oxygen at room temperature with excess CO_2 scavenged using Sofnolime soda lime chips (Molecular Products, UK) with colour indicator. In models of acute hypoxia, LPS stimulated or naïve mice were put into hypoxia for 2-48 hours prior to tissue harvest.

LPS Induced acute lung injury

Mice were treated with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* 10 (1mg/ml, Sigma Aldrich) for 10 minutes using oxygen driven nebulisation to generate an acute lung injury. Following LPS treatment, animals were housed in either normal room air or in hypoxia. Animals were culled using an overdose of intraperitoneal (IP) anaesthetic at the appropriate time point (2-48 hours post-nebulisation) and tissues harvested.

Intraperitoneal chloroquine administration

Following administration of nebulised LPS, as described above, mice were treated with intraperitoneal (IP) chloroquine diphosphate (Sigma Aldrich) at a dose of 50mg/kg diluted in PBS. Control mice were given IP PBS alone. Administration of chloroquine was either immediately following LPS treatment or four hours following LPS treatment and/or exposure to hypoxia, as detailed in the results section. Following chloroquine or PBS injection, animals were housed as before in either normoxia or hypoxia until being culled by overdose of intraperitoneal anaesthetic 24 hours post-LPS.

Isolation of murine neutrophils

Blood neutrophils:

Following overdose of anaesthetic, the peritoneal cavity was dissected, and blood withdrawn from the inferior vena cava (IVC) using a 21G needle and 1ml syringe. For neutrophil functional assays the syringe was heparinised prior to blood collection and for flow cytometry 30μl of 5mM EDTA was added to the collection vessel for anticoagulation. For all experiments red blood cell lysis was carried out as follows: 1ml of red blood cell lysis buffer (Biolegend) was added per 100μl of whole blood in a 15ml falcon tube and incubated at room temperature for 5 minutes then topped up to 15ml with PBS. Samples were centrifuged for 5 minutes at 350G, the supernatant discarded, and the lysis step repeated followed by washing in PBS. Blood neutrophils were purified for functional assays using the EasySep negative magnetic selection kit (Stemcell technologies). A detailed protocol is in Appendix 2. In brief, the samples were blocked with rat serum to reduce non-specific binding, an antibody cocktail which binds the unwanted leucocytes with biotinylated antibodies was added and, following incubation, cells were washed and resuspended with a biotin selection cocktail and then magnetic beads which bind the antibody complexed cells. The unwanted cells were then removed by incubation on a plate magnet. Following neutrophil isolation cell counts were assessed by haemocytometer and purity by morphology on cytospin. Using this technique, purities of 85-95% were achieved.

Bronchoalveolar lavage (BAL) neutrophils:

BAL cells were isolated after collection of venous blood. Following dissection of the peritoneal cavity the diaphragm was exposed and punctured to induce a pneumothorax. The diaphragm and thoracic cavity were then dissected to expose the lungs. The trachea was cannulated using a 24G cannula which was tied in place. The lungs were washed with ice cold 0.9% NaCl (5 aliquots of 0.8ml to give a total of 4ml) and BAL fluid kept on ice until further processing. Cell counts were carried out using a haemocytometer and purity assessed by morphology on cytospin.

Highly pure BAL neutrophils

A discontinuous percoll gradient was used to purify BAL neutrophils for RNA, protein and metabolite assays. The protocol is similar to that described for human blood neutrophil isolation but was optimised for murine BAL cells: Percoll densities were adjusted (as detailed in Appendix 1) and the gradient was centrifuged for longer and at a higher speed. BAL cells were first pelleted by centrifugation at 350G for 10 minutes. BAL samples from 1-3 mice (depending on the final cell number required) were pooled onto a single percoll gradient. 3ml of 78% percoll was pipetted into the bottom of a 15ml falcon tube, followed by 3ml of 69% percoll. The cell pellet(s) were resuspended in 3ml of 52% percoll which was then layered onto the 69% layer. The gradient(s) were centrifuged at 1200G for 30mins with an acceleration of 1 and deceleration of 0. Following centrifugation, the macrophages accumulated on the top of the 52% layer and were removed using a sterile pastette. The neutrophils (within the 69% layer) was then removed using a fresh sterile pastette and placed into

a 50ml polystyrene falcon tube. This was topped up to 50ml with sterile PBS to wash off the percoll and the purified neutrophils pelleted at 350G for 8 mins. Residual red cells were lysed by hypotonic saline lysis: The pellet was resuspended in 7ml of 0.2% NaCl and incubated at room temp for 5 mins prior to adding 7ml of 1.6% NaCl and pelleting the cells by spinning at 350G for 8 minutes. The pure neutrophils were then counted by haemocytometer prior to storage or lysis as appropriate. A cytospin was made prior to the final centrifugation step to assess purity by morphology. During optimisation of this protocol, purity was also confirmed by flow cytometry and the neutrophils were found to be >98% pure.

Bone Marrow Neutrophils:

A discontinuous percoll gradient was also used to isolate bone marrow neutrophils. Details of the reagents described and percoll preparations are in Appendix 1. Both hind limbs were dissected out and stored in a clean bijou on ice until processed. The limb was dislocated at the knee and ankle and muscle and connective tissue removed from the femur and tibia. Each bone was flushed using 10ml of 1xHBSS with 0.2% BSA. The cell suspension was resuspended using a pastette and passed through a 70μm filter into a fresh 50ml polystyrene falcon tube and then centrifuged at 450G for 10 minutes. 90% vol/vol percoll stock was made using 10X HBSS with $NaHCO_3$ and this stock was used to make 81%, 62% and 55% percoll with 1x HBSS (as detailed in Appendix 1). All three percoll preparations were layered in a 15ml falcon tube and the cells were resuspended in 3ml of 1xHBSS with 0.2% BSA which was layered on top of the 52% percoll layer. The gradient was centrifuged at

2000G for 30 minutes (acceleration 1, deceleration 0). Bone marrow monocyte precursors were removed from the top layer with a pastette and cultured as required. Neutrophils were removed from the middle layer and washed in 30ml of 1xHBSS with 0.2%BSA. Hypotonic saline red blood cell lysis was carried out as described above in the highly pure BAL neutrophil protocol.

Highly pure bone marrow neutrophils

Following the percoll purification above, neutrophil purity was found to be approximately 90%. In order to generate highly pure bone marrow neutrophils samples, cells from the neutrophil layer were resuspended in 1xHBSS with 0.2% BSA and purified by fluorescence-activated cell sorting (FACS) based on the forward/side scatter profile and auto-fluorescence as previously described[112]. Cells were collected into 1xHBSS with 0.2% BSA and washed with PBS prior to downstream applications. Using this technique, purities of >98% were consistently achieved (as analysed by flow cytometry of sorted cell populations.

Murine Lung Digest

Following the BAL protocol described above, the right ventricle was cannulated, and the circulation flushed with 10ml of ice-cold PBS to minimise circulating leucocytes within the lung vasculature prior to harvest. Both lungs were dissected out and any associated tissues removed before the digest protocol. The lung tissue was mechanically dissociated using scissors prior to enzymatic digestion with the following cocktail made up in RPMI:

Enzyme	Working concentration	Source	Product code
Collagenase V	0.8mg/ml	Sigma-Aldrich	C9263-1G
Collagenase D	0.625mg/ml	Roche	11088882001
Dispase	1mg/ml	Gibco	17105-041
DNAse	30μg/ml	Roche	101 104 159 001

Whole lung tissue was incubated in 2ml of the enzyme cocktail at 37°C for 45 minutes with continuous shaking and underwent vigorous manual shaking every 10 minutes during the digest step. The digest was then passed through a 100μm filter into a 50ml polystyrene falcon tube and the plunger of a 5ml syringe was used to disrupt any remaining clumps of tissue. The filter was rinsed through with PBS and the sample topped up to 50ml. The cell suspension was pelleted by centrifugation at 300G for 5 minutes at 4°C. The pellet was resuspended in 1ml of red cell lysis buffer (Sigma Aldrich) for 2 minutes at room temperature before topping up to 50ml with PBS and centrifuging again at 300G for 5 minutes at 4°C. The pellet was then resuspended in 5ml of FACS buffer (PBS with 5%BSA and 2mM EDTA) and passed through a 40μm filter. The cell suspension was counted using a NucleoCounter automatic cell counter (Eppendorf) and after centrifugation at 300G for 5 minutes, resuspended at a density of $1x10^6$cells/ml. An aliquot of $2x10^5$ cells was then processed for flow cytometry analysis.

Cell Culture

Neutrophils

For all experiments, murine neutrophils were cultured at a density of $1x10^6$/ml and human neutrophils at a density of $5x10^6$/ml. Media was supplemented with 1% Penicillin Streptomycin (Gibco) and 10% dialysed FCS (Gibco) unless otherwise stated. Neutrophils were cultured in RPMI 1640 media with 2mM glutamine (Gibco). The glucose replete media had 11mM D-glucose.

Standard cell culture was carried out in a Thermo Scientific Heraeus BB15 CO2 incubator. For hypoxic cell culture, conditions were as above but cells were cultured in a Ruskin hypoxic work station (Ruskin, UK) with an O_2 of 1% and CO_2 of 5%. Humidity in the hypoxic work station was maintained at 70%.

Human embryonic kidney (HEK) 293 Cells

HEK cell lysates were used as a substrate to analyse the downstream use of scavenged proteins. This human cell line was generated by transfection of human embryonic kidney cells with adenovirus fragments[113]. HEK 293 cells were a gift from Alex Von Kriegsheim (IGMM, University of Edinburgh). Control cells were cultured in glucose replete DMEM (Gibco) with 10% dialysed FCS, 1% Penicillin Streptomycin and 2mM L-glutamine. For glutamine labelling experiments, standard L-Glutamine was substituted with $^{13}C_5$ labelled Glutamine (Cambridge Isotopes, USA) for 72 hours prior to being harvested and lysed.

Acetone precipitation of HEK cell proteins

Cells were harvested by washing with ice-cold PBS. The cells were then washed three times with fresh PBS to remove any residual media. The cell

pellet was then lysed by hypotonic lysis in 4ml of sterile water prior to sonication at 4°C for 10 minutes. The lysate was then divided into aliquots for acetone precipitation of protein as follows: The acetone was pre-cooled to -20°C and then added to the sample at a ratio of 4:1 by volume. The tube was vortexed and then incubated at -20°C for 60 minutes. The sample was then centrifuged at 13,000G for 10 minutes and the supernatant (containing small molecules such as metabolites and amino acids) was discarded. Residual acetone was allowed to evaporate by holding the uncapped tube at room temperature for 30 minutes. The pellet was resuspended in PBS. Some residual insoluble protein material remained and so the sample was centrifuged at 13,000rpm for 10 minutes to pellet this material and the supernatant utilised for downstream culture. Protein content was measured using a BCA assay (Thermo Scientific) and normalised between labelled and unlabelled samples.

Ex vivo culture of BAL neutrophils with labelled proteins

BAL neutrophils were isolated and purified by percoll gradient as detailed above. Following purification neutrophils were resuspended in glucose and glutamine free DMEM (Gibco) supplemented with 10% FCS and 400μg/ml of HEK cell protein isolated by acetone precipitation as above. Neutrophils were cultured at a density of $1x10^6$/ml for 18 hours in hypoxia. Following this period of culture, cells were washed three times with sterile 0.9% NaCl prior to methanol lysis as detailed below.

Bone marrow derived monocytes

Bone marrow derived monocytes (BMDMs) were generated by taking the top layer of cells from the percoll gradient (described above). Cells were counted by haemocytometer and resuspended at $3x10^5$/ml in DMEM/F-12 Glutamax media (Gibco) with 10% FCS and 1% Penicillin Streptomycin. Media was supplemented with 20% L929 conditioned media (as a source of M-CSF) for the first 7 days of culture. On day 7 cells were washed and then harvested using acutase and after washing in PBS were resuspended in RNA lysis buffer. An aliquot was retained to analyse differentiation by flow cytometry based on staining for the monocyte marker F4/80.

Sample preparation for proteomic analysis

The workflow for proteomic sample generation is illustrated in Figure 2.2 and details of the lysis buffers used are in Appendix 3. 1 x10^6 highly purified BAL neutrophils were lysed in SDS lysis buffer with TCEP at a10mM working concentration and boiled for 5 minutes. Samples underwent 15 minutes of sonication then incubation with benzonase for 15 mins at 37°C. Protein concentration was estimated using the EZQ protein quantitation kit (Invitrogen) as per manufacturer instructions. Following quantification, iodoacetamide (IAA) was added at a working concentration of 20mM and lysates incubated for 1 hour at room temperature. In-solution digestion was carried out using SP3 beads and LysC (overnight incubation at 37°C) at a concentration of 1μg LysC (Promega) per 100μg protein followed by trypsin (Promega) digestion, also at 1μg enzyme per 100μg protein overnight at 37°C. Peptides were eluted using acetonitrile (ACN) and quantified using the CBQCA assay. Samples were diluted to a concentration of 1μg/15μl with 5% formic acid and 1μg of peptide was submitted for single shot analysis or fractionation. During optimisation experiments, single shot analysis of BAL neutrophils recovered approximately 2000 proteins, while fractionation prior to analysis yielded over 5000 proteins, the fractionation strategy was therefore used for all samples. Samples were fractionated using strong anion exchange chromatography, analysed in an LTQ-Orbitrap Velos MS, and raw data processed using MaxQuant software[114]. Subsequent statistical analysis and figure generation was carried out using either Perseus software[115], Microsoft Excel or GraphPad Prism.

Figure 2.2

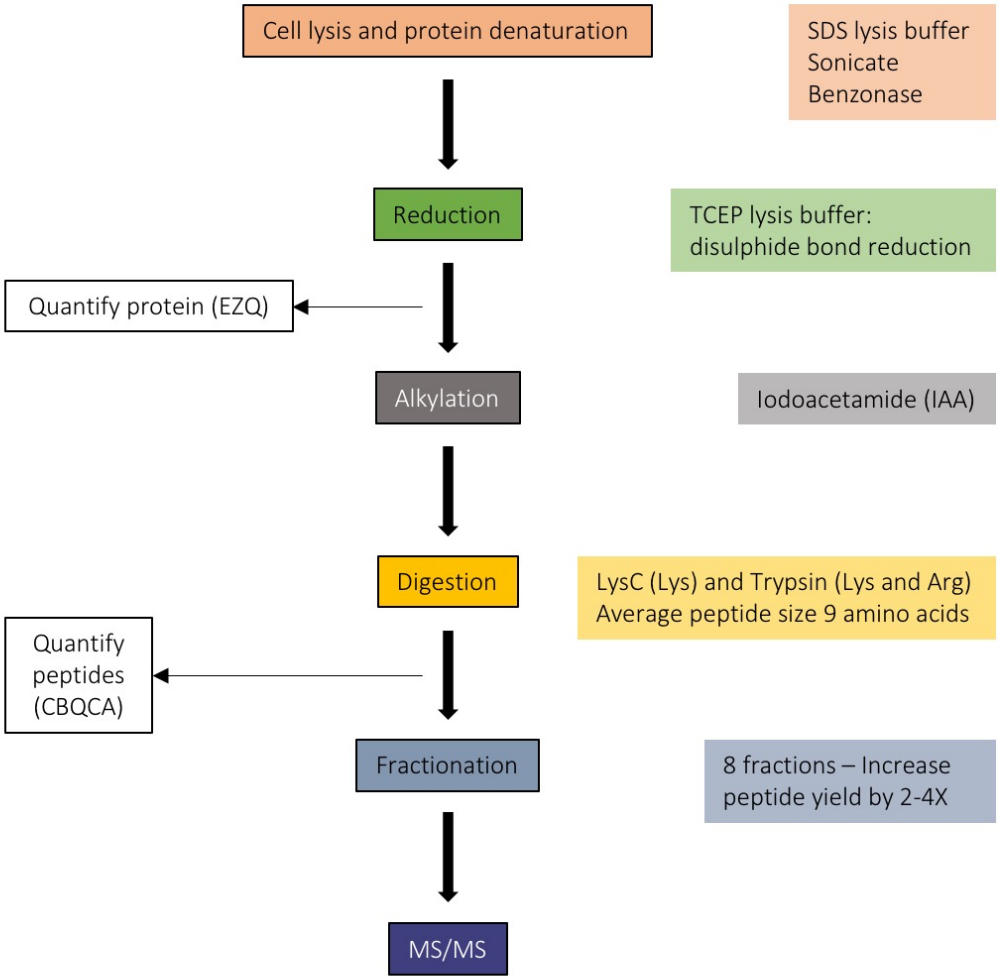


Figure 2.2: Proteomic sample processing
Following purification, BAL neutrophils underwent cell lysis. Proteins were then reduced, alkylated and digested using LysC and trypsin. HPLC fractionation improved the yield of identifiable proteins. Samples were analysed by mass spectrometry.

Neutrophil protein lysates

Neutrophils possess high levels of proteases necessitating careful lysis with strong protease inhibition to avoid breakdown of proteins of interest. In order to allow measurement of phosphorylated proteins in neutrophil protein lysates the lysis protocol was further optimised to ensure preservation of phosphorylated proteins. Protocols for making the buffers described are included in Appendix 3. For human peripheral blood neutrophils, 5-10 million neutrophils were used per sample and for murine protein lysates, 1 million neutrophils were used per sample.

Isolated neutrophils were washed twice with cold PBS following culture or purification. Cells were pelleted by centrifugation at 300G for 5 minutes and the supernatant removed. The pellet was resuspended in Laemmli buffer with PMSF, phosphatase inhibitors and protease inhibitors (100μl Laemmli buffer per 1 million murine or 10 million human neutrophils). An equal volume of 2x SDS lysis buffer was then added and mixed by pipetting. The sample was then boiled for 5 minutes prior to storage at -80°C until use.

Immunoblot for protein expression

Neutrophil protein lysates were analysed for protein expression by western blot. Details of the gels and buffers used are in Appendix 4. Proteins were separated by SDS-PAGE using the BioRad mini-protean system prior to wet transfer onto PVDF membrane (Merck Millipore). Membranes were blocked using 5% skimmed milk powder for a minimum of 2 hours. Primary antibodies were made up in 5% skimmed milk in 1X TBS-Tween (TBST, 0.05% Tween) and membranes incubated at 4°C overnight on a rolling platform. Details of

primary antibodies and their working concentrations are in Appendix 4. Membranes underwent three 10-minute washes in 1X TBST prior to incubation with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature. The membrane was washed as above prior to developing in enhanced chemiluminescent (ECL) detection reagent (GE Healthcare). Chemiluminescence was assessed using a Licor Odyssey Fc machine and quantified using ImageStudioLite software. Protein quantification was normalised to a loading control as follows: The membrane was stripped using Restore Western Blot stripping buffer (Thermo Scientific), washed with dH_2O and then re-probed for the loading control protein (or, for the non-phosphorylated form of the protein of interest where appropriate) with primary and secondary antibodies as above. The membrane was then developed, and protein quantified as above.

Neutrophil RNA extraction and TaqMan analysis of gene expression

The relative paucity of mRNA in neutrophils compared to other immune cells means that even a small percentage of contaminating cells can significantly influence the results of gene expression analysis and so in all experiments highly pure neutrophils were used to generate mRNA samples.

A minimum of $1x10^6$ neutrophils was required to generate sufficient quantity and quality of mRNA. The *mir*Vana miRNA isolation kit (Invitrogen) was used to isolate mRNA from samples. A detailed protocol is in Appendix 5. In brief, the cell pellet is resuspended in a denaturing lysis solution to stabilise RNA and inactivate RNAses. The RNA was extracted using an acid-phenol:chloroform step and then further purified by immobilisation on a glass-fibre filter prior to elution with water. The samples then underwent a DNase step to ensure removal of any contaminating genomic DNA using the Invitrogen TURBO DNA free kit (protocol in Appendix 5). The quantity and purity of the RNA was assessed using a Nanodrop 100 spectrophotometer.

The quantity of RNA used to generate cDNA was dependent on the nature of the samples and ranged from 250ng to 1μg. Samples for direct comparison were always normalised to generate the same quantity of cDNA and were made up to 12.4μl in RNase-free water. cDNA was made using AMV reverse transcriptase and random primers(Promega), details of the master mix used are in Appendix 6. Samples were run on a Techne Thermal cycler as follows:

- 23°C for 5 minutes
- 42°C for 2 hours
- 99°C for 2 minutes (to heat inactivate AMV RT)

Samples were stored at -20°C until use.

Gene expression was analysed using predesigned qPCR primer/probe assays (detailed in Appendix 6) and Prime Time Gene Expression Mastermix (IDT, Leuven). For all assays, samples were run in triplicate and the gene of interest expressed relative to expression of a housekeeping gene (beta-actin). Assays were run on a 7900HT Fast real-time PCR system (Applied Biosystems) with the following negative controls: no RNA, no RT and no cDNA. Data was analysed using SDS 2.0 software (Thermo Scientific).

Analysis of metabolites

HPLC-MS analysis of metabolites

Highly pure BAL neutrophils were analysed for metabolite abundance. Following percoll purification as described, cells were washed in ice cold 0.9% NaCl prior to being pelleted (300G for 5 minutes). The pellet was lysed by resuspension in 80% Methanol which had been stored overnight at -80°C and kept on dry ice during resuspension. Prior to analysis, the lysed sample was centrifuged at 20,000G for 10 minutes at 4°C and the supernatant removed into a fresh Eppendorf tube. The pellet was retained, and protein content measured by Pierce BCA assay (Thermo Scientific). Samples were analysed at the VIB Center for Cancer Biology in Leuven using a Dionex UltiMate 3000 LC System (Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) operated in negative mode. Data collection was performed using Xcalibur software (Thermo Scientific). All data values were subsequently corrected for protein content based on the BCA assay.

Measurement of intracellular glycogen stores

BAL neutrophils were isolated 24 hours following LPS induced lung injury. $1.5x10^6$ highly pure BAL neutrophils cells were lysed in ice cold water and then boiled for 5 minutes. The lysate was centrifuged at 13,000G for 5 minutes and the supernatant used for measurement of intracellular glycogen using a colorimetric plate assay (Sigma Aldrich). The supernatant was used at a 1:2 dilution and the assay run as per the manufacturer's protocol.

Murine BAL supernatant analysis

The first 0.8ml aliquot of BAL fluid was used to analyse the activity or quantity of a number of proteins and metabolites within the BAL fluid. The freshly isolated aliquot was kept on ice and centrifuged at 2000rpm for 10 minutes at 4°C to pellet the BAL leucocytes and erythrocytes present in the aliquot. The supernatant was removed into a fresh tube, flash frozen on dry ice and stored at -80°C until use. In all assays commercial kits were used as per the manufacturer protocol and the optimal dilution of samples was ascertained for each different analyte. The optimised dilutions were as follows and product details are in the Appendix 11

	Dilution of BAL Fluid
Activity assays	
Elastase	Neat
Myeloperoxidase	Neat
Quantification assays	
Albumin	1:100000
Alpha-1 antitrypsin	1:200
Glucose	Neat
Glutamine	Neat*
GM-CSF	Neat
IgM	1:20
MMP9	1:400

*following deproteinization using 25kDa spin column

Analysis of murine neutrophils by flow cytometry

Murine whole blood

300-500μl blood was collected into 30μl EDTA and underwent red cell lysis as described above. Cells were counted using a haemocytometer and $2x10^5$ cells were stained for flow cytometric analysis

Murine BAL

Whole BAL was counted by haemocytometer and resuspended at a density of $1x10^6$. An aliquot of $2x10^5$ cells were then stained for analysis.

Murine lung digest

Following digest as described above, $2x10^5$ cells were stained and analysed by flow cytometry.

Murine flow cytometry staining protocol:

Viability assay: For lung digest samples only, samples were first resuspended in Live/Dead fixable Aqua stain (Invitrogen) as per manufacturer's protocol.

For all tissue types, samples were blocked in 25μl of PBS with 10% mouse serum and 1% Fc Block (Biolegend) for 15 minutes on ice. The antibody master mix was made up in PBS (with the exception of intracellular staining, see below) and 50μl of master mix was added to each sample. Antibody concentrations and fluorophore details are in Appendix 7. Cells were stained for 30 minutes on ice in the dark prior to being washed three times in FACS buffer and either being fixed in 4% PFA for 15 minutes at room temperature or analysed immediately on a BD LSR Fortessa cell analyser. For all flow cytometry experiments, data was acquired using FACS Diva software and analysed using FlowJo software (version 10.1).

Intracellular staining of BAL neutrophils for flow cytometry

Whole BAL was first prepared as above and incubated with an anti-Ly6G antibody to allow identification of the neutrophils on subsequent flow cytometry. Samples were washed twice with PBS prior to staining for intracellular markers. A commercial "cytofix/cytoperm" kit was used for this assay (BD Biosciences) to improve intracellular staining. Cells were resuspended in 200μl of fixation/permeabilisation solution for 20 minutes at 4°C. All wash and subsequent antibody incubation steps were carried out in 1x Perm/Wash buffer to maintain permeabilisation (this wash solution contains FBS and saponin). Following fixation, samples were washed and resuspended in 50μl of Perm/Wash buffer containing the fluorophore conjugated primary antibody. Samples were incubated for 30 minutes at 4°C and then washed twice. Samples were then resuspended in FACS buffer and analysed as detailed above.

Neutrophil Functional Assays

Phagocytosis

Phagocytosis was assessed by uptake of fluorescently labelled, heat killed E Coli. BAL neutrophils were isolated as above, resuspended at $1x10^6$ cells/ml and incubated at 37°C for 1 hour in RPMI with 10% FCS with the bacteria at a multiplicity of infection (MOI) of 1. For each sample an ice control was run to account for extracellular adhesion of bacteria. In this case cells were incubated as above but in an iced water bath for 1 hour. All samples were washed 3 times with ice cold PBS prior to being resuspended in FACS buffer and analysed by flow cytometry.

Chemotaxis

Chemotaxis assays were performed on murine BAL neutrophils isolated following LPS induced lung injury. Whole BAL was counted and resuspended at $2x10^6$cells/ml. The assay measured movement of cells across a filter towards a chemoattractant (KC, also called CXCL1, for murine neutrophils) using the ChemoTx plate system (Neuro Probe). A positive control, chemokinesis control and negative control were run with each assay and a dose response was measured by using different concentrations of the chemoattractant. An example plate plan is in Appendix 8. The chemoattractant or control was pipetted into the bottom well of the plate, the filter was then placed on top and the cell suspension pipetted onto the filter. The plate was incubated at 37°C for 1 hour with a lid on. Residual cell suspension was removed from the top of the filter using a cotton bud and the plate centrifuged (without the lid) for 10 minutes at 300G. The filter was removed and the cellular

concentration (i.e. the number of cells which have migrated across the filter) was measured in each well using a haemocytometer count of the resuspended well contents. This number was adjusted for the volume in each well to give an absolute cell count.

Respiratory burst assay

Neutrophil respiratory burst was measured by a chloromethyl derivative of 2',7'-Dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Invitrogen). Upon oxidation H2DCFDA becomes the green fluorescent 2',7'-Dichlorofluorescein. The dye is cell permeable and passively enters the cell. Upon oxidation it emits a green fluorescent signal which can be measured by flow cytometry. The baseline oxidative stress and respiratory burst capacity of BAL neutrophils was measured as follows:

CM-H2DCFDA was resuspended in at 50μg/5μl of DMSO. This was then diluted 1:100 with PBS to make up the stock solution. 3μl of this stock solution was added to 100μl of BAL neutrophils at $1x10^6$/ml and cells were incubated at 37°C for 45 minutes. To measure respiratory burst capacity, the formylated peptide F-met-leu-phe (fMLF, Sigma Aldrich), a potent stimulator of neutrophils, was added after 45 minutes for a further 45 minutes at a final concentration of 10μM. Following this second incubation the samples were analysed by flow cytometry (maximum excitation/emission: ~492-495/517-527nm).

Measurement of neutrophil apoptosis

Neutrophils display characteristic nuclear morphology both when viable and when undergoing apoptosis allowing the extent of apoptosis to be analysed by nuclear morphology on a cytospin with light microscopy. Alternatively, apoptosis may be quantified using flow cytometry techniques. These exploit one of two key events which occur during apoptosis and if used in combination can distinguish early and late apoptosis. Annexin V binds phosphatidylserine (PS) residues which are externalised (and thus available to bind to Annexin V) early in the process of apoptosis. Annexin V is conjugated to a fluorophore to allow identification of PS positive cells by flow cytometry. Cells dying by both apoptosis and necrosis will undergo a loss of membrane integrity. TO-PRO-3 stain is a cell impermeable dye which binds double stranded DNA. Once the plasma membrane is compromised during cell death, TO-PRO-3 can bind the, now accessible, DNA and emits far-red fluorescence. Thus, by dual staining with both Annexin V and TO-PRO-3 cells can be identified as viable (double negative), early apoptotic (Annexin V positive), late apoptotic (double positive) or necrotic (TO-PRO-3 positive).

Apoptosis by morphology

Following culture and/or treatment, a cytospin was generated. Apoptosis was assessed by morphology and 300 neutrophils in total are counted to ascertain the percentage of apoptosis for each cytospin.

Apoptosis by flow cytometry

Following culture and/or treatment, cells were washed in PBS prior to staining. 1X Annexin binding buffer (BD Biosciences UK) was made and cells were

resuspended in 100μl of 1x binding buffer with Phycoerythrin (PE) conjugated Annexin V at a dilution of 1:25. Samples were incubated on ice for 20 minutes. TO-PRO-3 stain was made up at a 1:5000 dilution with 1x Annexin binding buffer. Immediately before running the sample on the flow cytometer, 100μl of diluted TO-PRO-3 was added to the sample.

Cell counts with flow cytometry

In order to measure cell loss in conjunction with flow analysis of apoptosis, CountBright beads (Invitrogen) were used to provide an accurate total cell count. 10μl of bead suspension was added to each sample prior to the addition of TO-PRO-3 stain. These 7μm beads are provided in a suspension at a known concentration and are fluorescent across multiple channels. They can therefore be gated on and counted by the flow cytometer and the ratio of cells to beads used to calculate the cellular concentration of the sample.

Flow cytometry was carried out on a BD LSR Fortessa cell analyser. Data acquisition used FACS Diva software and data was analysed using FlowJo software.

Dextran and albumin uptake assays

In order to investigate the capacity of neutrophils to take up proteins and other substances from the extracellular environment, uptake assays were carried out using three different compounds:

70kDa dextran has been shown to be taken up exclusively via fluid phase uptake, i.e. macropinocytosis. Neutrophil macropinocytosis was measured by culturing neutrophils in media supplemented with Texas-Red labelled 70kDa dextran (Invitrogen) at a final concentration of 1mg/ml. Samples were then washed once in ice-cold citric acid (pH5.5) then twice with cold PBS. Uptake was quantified by flow cytometry and localisation assessed by confocal microscopy (see below).

Fluorescently labelled BSA (Invitrogen) was used to measure neutrophil albumin uptake. Neutrophils were cultured in complete media with FITC or Texas-Red labelled BSA (Invitrogen) at a final concentration of 200μg/ml.

Double-quenched (DQ) Green BSA (Invitrogen) was used to measure proteolytic cleavage of BSA taken up by neutrophils. This compound is a derivative of BSA which is labelled with sufficient BODIPY dye that the dye is self-quenching. Upon proteolytic cleavage, a single, BODIPY-labelled albumin peptide is released and is de-quenched, resulting in a fluorescent signal. Importantly, this signal in independent of pH. For both the fluorescent and DQ labelled BSA, samples were washed as detailed above for the dextran assay and fluorescence measured by flow cytometry and localisation analysed by confocal microscopy.

In order to assess the effect of hypoxia and glucose deprivation on albumin uptake and breakdown, neutrophils were pre-incubated in either normoxia or hypoxia, in glucose deplete or replete media for 2 hours prior to the addition of the fluorescently labelled compound (± LPS at a final concentration of 100ng/ml).

Lysosomal inhibitors were used to treat unstimulated neutrophils as follows: Following two hours of culture (to equilibrate to culture conditions), the protease inhibitor E64 or chloroquine (Sigma-Aldrich) were added at a final concentration of 2μM and 10μM respectively, both for 1 hour prior to fluorescent albumin being added.

The mTOR inhibitor rapamycin (Sigma-Aldrich) or the activator of mTOR MHY1485 (Merck chemicals) was added to neutrophils at final concentrations of 50nM and 2μM respectively. As before, cells were equilibrated to culture conditions for 2 hours first and the inhibitor was added for 1 hour prior to the addition of LPS and fluorescent albumin. For both the lysosomal inhibitor experiments and the mTOR modulating experiments, fluorescent albumin products were added for 90 minutes and the cells were subsequently washed and run on flow cytometry as before.

Confocal microscopy of neutrophils

Confocal microscopy was used to delineate the intra-cellular location of the fluorescently labelled compounds described above. Following culture with fluorescent compounds, neutrophils were washed with ice-cold PBS three times. Neutrophils then underwent fixation and permeabilisation prior to additional staining. Two different strategies were used for this process:

1. *Co-localisation of dextran and BSA:* Cells were cultured with Texas Red dextran and FITC-BSA simultaneously (as detailed above). The cells were then washed and fixed in 4% PFA for 15 minutes at room temperature followed by permeabilisation with 0.1% Triton X100 for 15 minutes at room temperature. The cells were washed again in PBS before staining with a deep-red cell mask (Invitrogen) by resuspending the washed cells in 200μl of cell mask at a final concentration of 1μg/ml for 30 minutes at room temperature.
2. *LAMP1 staining for colocalization of DQ-Green signal with the lysosome:* A commercial "cytofix/cytoperm" kit was used for this assay (BD Biosciences) to improve intracellular staining. Cells were resuspended in 200μl of fixation/permeabilisation solution for 20 minutes at 4°C. All wash and subsequent antibody incubation steps were carried out in 1x Perm/Wash buffer to maintain permeabilisation (this wash solution contains FBS and saponin). The primary antibody was a mouse monoclonal antibody to human LAMP1 (Abcam) and cells were stained for 1 hour at a final antibody concentration of 10μg/ml before being washed twice and stained with an Alexa Fluor 647

conjugated goat anti-mouse secondary antibody (Abcam) used at 1:200 (50μl per sample) for 1 hour at room temperature.

For both strategies, cells were washed twice following the final staining step and the pellet resuspended in ProLong Gold liquid mountant with DAPI for nuclear staining (Invitrogen). This suspension was pipetted (with care to avoid bubbles) onto a glass slide and covered with a glass coverslip. Slides were left to dry for at least 24 hours in the dark. Confocal images were acquired using a Leica SP5 confocal laser scanning microscope with Leica Application Suite software.

Chapter 3: Systemic Hypoxia Results in a Hyperinflammatory Neutrophil Phenotype

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are clinical syndromes characterised by lung inflammation, increased vascular permeability, and hypoxaemia[116]. ALI and ARDS represent a continuum with more severe hypoxia defining ARDS. ALI and ARDS are associated with significant morbidity and mortality (a meta-analysis of published data found the average mortality rate for ARDS was 43%)[117]. In addition to being a defining feature of ALI and ARDS[118], the degree of hypoxia is also associated with negative outcomes, including increased alveolar damage[119] and increased mortality[120]. Neutrophil infiltrates are characteristic of ARDS and the extent and persistence of lung neutrophilia are correlated with increased mortality[121]. Hence, the impact of hypoxia on neutrophilic inflammation is an important consideration in this disease.

The inflammatory response is a dynamic process, in functional immunity this is characterised by onset of inflammation (due to infection or injury), recruitment and activation of immune cells, removal of the pathogen or healing of the injury and resolution of the inflammatory response. Neutrophils play an important part in initiation of immunity due to their capacity to move rapidly to sites of injury and control the invading pathogen through multiple antimicrobial tools. Neutrophils also facilitate induction of adaptive immunity. In viral infections such as Influenza, infected neutrophils are able to act as antigen

presenting cells (APCs) to $CD8^+$ T lymphocytes[122]. Neutrophils are also an important source of inflammatory cytokines and chemokines which have been shown to recruit dendritic cells (professional APCs) to the site of infection[123]. Timely resolution of the inflammatory response is essential to restore homeostasis. Neutrophils which have been recruited to the site of infection or inflammation must be cleared to avoid release of further inflammatory mediators and perpetuation of the inflammatory response. Apoptosis or programmed cell death is an important step in resolution of neutrophilic inflammation[22]. Neutrophils undergoing apoptosis become unresponsive to inflammatory stimuli, stop releasing inflammatory mediators and are subject to uptake by macrophages (efferocytosis) [124] which in turn, polarises a pro-resolution macrophage phenotype. Failure of neutrophils to undergo apoptosis may therefore result in persistent inflammation and significant tissue damage. In order to understand regulation of the neutrophil inflammatory response, we must therefore consider each stage of the response because the sum of all these events will determine the overall outcome.

Neutrophils are the most abundant leucocyte in the circulation. They are therefore poised ready to move in response to danger signals in the tissues. Neutrophil recruitment typically occurs by transendothelial migration in the post-capillary venule. At sites of inflammation this is mediated by regulation and activation of a series of receptors present on the neutrophil surface. Additionally, activation of the endothelium in response to infection or injury results in upregulation of receptors and ligands which facilitate recruitment and migration of neutrophils. Both neutrophils and endothelial cells express

Selectins and their carbohydrate ligands. Selectin-ligand interactions allow tethering and rolling of neutrophils. This is initially a reversible interaction but additional activation of neutrophils (for example by chemoattactants or inflammatory stimuli) results in L-selectin shedding and upregulation of molecules involved in firm adhesion such as the integrin MAC-1 (a heterodimer of CD11b and CD18) which binds intracellular adhesion molecule (ICAM)1[125]. Subsequent crawling of neutrophils in response to chemokine signalling is followed by extravasation via transendothelial migration. Both the architecture and vascular supply to the lung have resulted in specific adaptations to neutrophil recruitment in this organ. The more proximal airways are supplied by the bronchial arterial circulation and neutrophils migrate from these vessels as described above. Meanwhile, the pulmonary circulation, whose primary role is in gas-exchange, predominates in the distal airways and alveolus. The alveolar capillaries are small in diameter, often smaller than the circulating neutrophils[126]. This has two important consequences, firstly neutrophils are required to deform from their usual spherical shape in order to pass through narrow capillaries. Secondly, neutrophil progress through the pulmonary vasculature is slowed down resulting in a pool of so-called marginated neutrophils within the pulmonary circulation[127]. This slowing is also thought to obviate the need for a rolling step in recruitment from these vessels. Neutrophil migration into the alveolus from the pulmonary circulation does not occur in the post-capillary venules but rather from the capillary bed and does not rely on the traditional rolling, firm attachment, crawling paradigm described above. The factors which regulate neutrophil transendothelial

migration in this context vary with the inflammatory stimulus but are likely still to be at least partially dependent on integrins and selectins[128].

Chemotaxis within the tissue is then mediated by further chemokine-receptor interactions. CXCR2 is an important chemokine receptor present on neutrophils. In the mouse, CXCR2 binds to the chemokines KC and MIP2 (also known as CXCL1 and CXCL2) and CXCR2 and its ligands have been found to be essential for neutrophil recruitment to the lung[129-131].

Upregulation of CD11b and shedding of CD62L (L-selectin) have been widely used as a measure of neutrophil activation status both in the circulation and in the lung[25,132]. Although, as the process of extravasation above describes, transmigration alone is likely to be associated with upregulation of CD11b and shedding of CD62L, indeed, in studies of patients with sarcoidosis[133] and asthma[134], both patients and controls showed higher CD11b and lower CD62L in lung compared with blood neutrophils. So, while surface expression of these molecules may provide some clues as to the activation status of neutrophils, more detailed analysis of neutrophil functions is required to fully understand their activity and capacity for host tissue damage.

In sterile inflammatory disorders, such as ARDS, the neutrophil antimicrobial arsenal has the capacity to cause severe host tissue damage. An important facet of the neutrophil antimicrobial response is the release of proteases, oxidant-producing enzymes and other molecules from granules upon appropriate stimulation. In order to facilitate rapid release of these proteins in response to infection, granules and their contents are preformed and packaged during neutrophil maturation in the bone marrow. This packaging

also serves to protect the neutrophil from the toxic granule contents[135]. Four different granule types are recognised, azurophil or primary granules, specific (secondary) granules, gelatinase or tertiary granules and secretory vesicles. Each has a specific repertoire of proteins, targeted to the appropriate granule subtype during granulopoiesis through a temporally regulated programme of transcription and translation[136]. Granules contain proteins which are released upon degranulation but also cell surface receptors in their inner membrane which, upon fusion of the granule membrane with the neutrophil plasma membrane, are presented on the cell surface. Degranulation is necessarily a highly regulated process as inappropriate release of these toxic proteins risks host cell and tissue damage. Neutrophil elastase is a serine protease present in azurophilic granules. Elastase release in the lung correlates with disease severity in cystic fibrosis[137] and serum elastase levels are increased in patients with COPD[138]. The serine protease inhibitor alpha 1-antitrypsin (encoded by the *serpinA1* gene) inhibits neutrophil elastase and alpha 1-antitrypsin deficiency is associated with development of emphysematous lung disease due to unopposed elastase mediated lung damage[139]. Regulation of neutrophil degranulation is therefore key in neutrophilic inflammation in the lung. Degranulation requires a coordinated intracellular response involving recruitment of granules to the plasma membrane, tethering of the granule membrane to the target membrane, fusion of the two membranes and release of granule contents. These processes require intracellular calcium signaling, cytoskeletal reorganization and activation of multiple intracellular signaling cascades. All of this occurs in

response to activation of various extracellular receptors including the formylated peptide receptors, GM-CSF receptors and toll-like receptors.

Hypoxia (which often accompanies respiratory disease) and inflammatory processes frequently co-exist. Neutrophils are required to function in markedly hostile environments where oxygen and nutrient availability are often low. As such, neutrophils are well adapted to survive and function in hypoxia. Neutrophils are unusual in that hypoxia is a profound survival stimulus for these, usually short lived, cells[21]. Key neutrophils functions including chemotaxis[140], and phagocytosis[141] are also preserved in hypoxic conditions. There is increasing evidence that hypoxia and hypoxic signaling pathways may result in enhanced neutrophilic inflammation. Hypoxia is known to increase neutrophil degranulation[142] and our group has shown that systemic hypoxia in the context of bacterial infection results in significant, neutrophil mediated, morbidity and mortality[143]. Furthermore, PHD2 inhibition or loss and subsequent HIF activation in myeloid cells resulted in exaggerated neutrophilic inflammation in response to *Streptococcus Pneumoniae* and in an LPS-induced acute lung injury model.

These data suggest that neutrophils display a degree of plasticity not previously recognised. The concept of polarisation in immune cells is well established in T cell and macrophage biology. It is increasingly apparent that neutrophils are a less homogeneous population than was previously thought. There is evidence that, in the context of the tumour environment, neutrophils can be polarised to an anti- or pro-tumour phenotype (so called N1 or N2 respectively) with TGF-β signalling favouring pro-tumour neutrophil

development[144]. More recently, treatment with a peroxisome proliferator-activated receptor γ (PPARγ) agonist was shown to favour an N2 neutrophil phenotype in neuronal inflammation following ischaemic injury and was associated with enhanced resolution of inflammation[145]. The nature of the neutrophil response is therefore heterogenous and is likely to be influenced by microenvironmental factors within the inflammatory milieu. I hypothesise that hypoxia, a common feature of inflammatory environments, will play an important role in shaping neutrophil responses. Given the evidence that hypoxia may enhance neutrophilic inflammation, I sought to define the mechanisms by which this occurs in the context of acute lung inflammation. I hypothesised that hypoxia results in a hyperinflammatory neutrophil phenotype with important consequences for inflammation and resolution.

Results

Hypoxia exacerbates acute lung injury, independent of neutrophil number

To investigate the role of hypoxia in regulating neutrophilic lung inflammation, I utilised a murine model of LPS-induced acute lung injury. This model of sterile inflammation produces a homogeneous and reproducible neutrophil mediated lung injury and allows study of the effect of hypoxia on host immune cells without the complication of hypoxic regulation of bacterial function associated with infection models. In the context of LPS induced ALI, concurrent exposure to hypoxia results in profound hypothermia over the first 24 hours (Fig 3.1A). Exposure to hypoxia in the absence of LPS nebulisation resulted in mild hypothermia but this resolved fully by 24 hours. This is a well-established model of neutrophilic inflammation and so it was interesting to note that the increasing sickness in hypoxia is not associated with increasing numbers of neutrophils in the BAL fluid for the first 24 hours (Fig 3.1B). However, by 48 hours post-injury, there is a trend towards higher neutrophil numbers in hypoxia, suggestive of impaired inflammation resolution (Fig 3.1B). At 24 hours post-LPS, there was also no difference in interstitial lung neutrophils (Fig 3.1C) and significantly fewer circulating neutrophils (Fig 3.1D).

In addition to the hypothermia observed in hypoxia, analysis of the BAL fluid supernatant also revealed increased lung injury with significantly increased albumin (Fig 3.2A) and IgM (Fig 3.2B) leak in hypoxia. Furthermore, there was evidence of increased neutrophilic inflammation with higher neutrophil elastase activity in hypoxic BAL supernatant, despite the similar numbers of recruited neutrophils (Fig 3.2C).

Figure 3.1

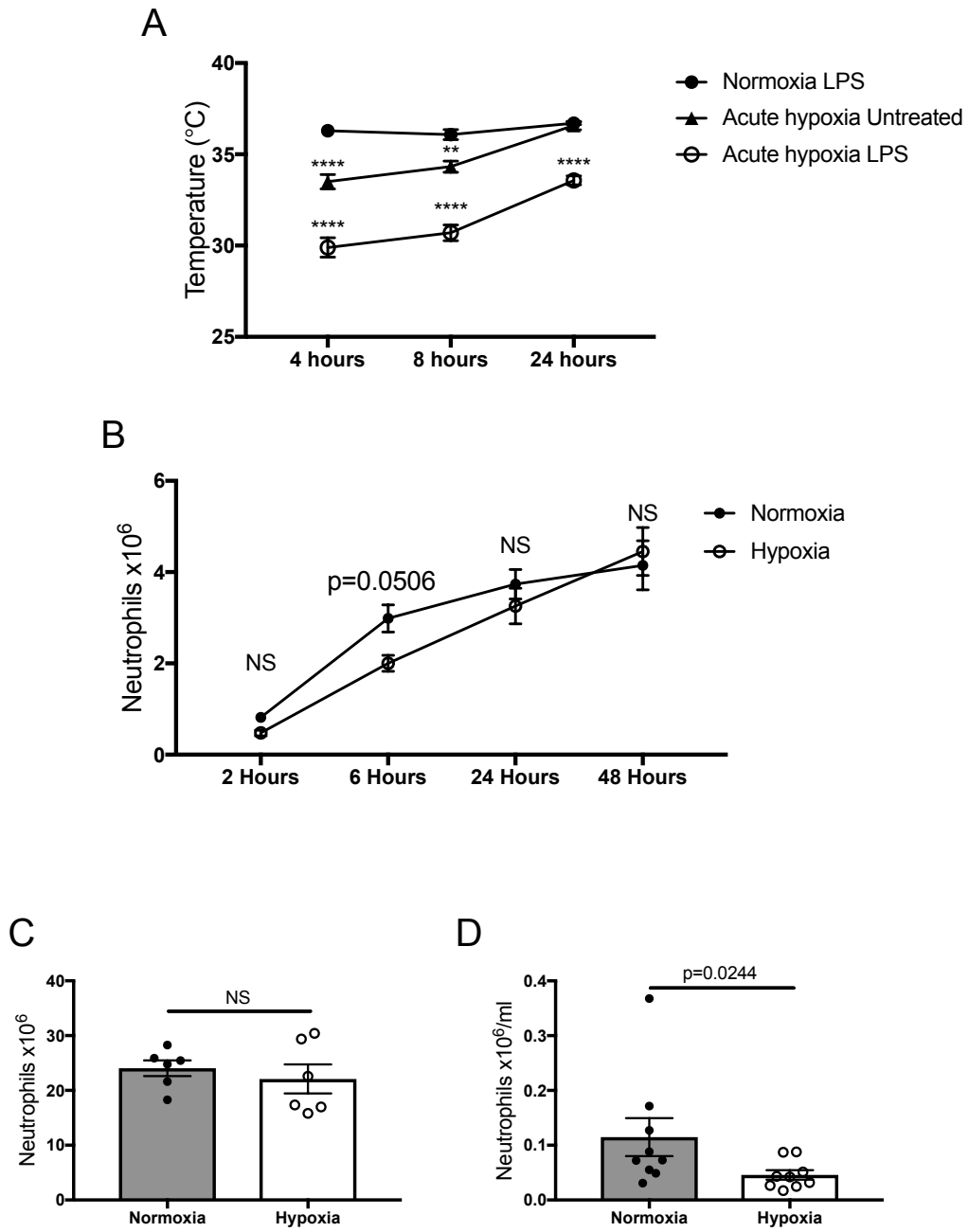


Figure 3.1: Hypoxia results in increased sickness without higher neutrophil numbers
(A) Body temperature of mice following nebulised LPS (black circles), hypoxia (10% O_2) alone (black triangles) and hypoxia following nebulised LPS (white circles), n=4. (B) BAL neutrophil counts of mice housed in either normal room air (normoxia) or hypoxia (10 %O_2) following nebulised LPS, n≥4. (C) Lung neutrophil counts and (D) blood neutrophil counts of mice housed in normoxia or hypoxia for 24 hours following LPS nebulisation, n≥6, data merged 2 (C) or 3 (D) experiments. (A) analysed by 2-way ANOVA with Tukey's multiple comparisons test, (B) significance analysed by multiple t-tests (corrected for multiple comparisons using Bonferroni method), * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. (C)&(D) Mann-Whitney test of significance. (A)&(B) Data represents mean ± SEM, (C)&(D) Data represents individual values and mean ± SEM.

Figure 3.2

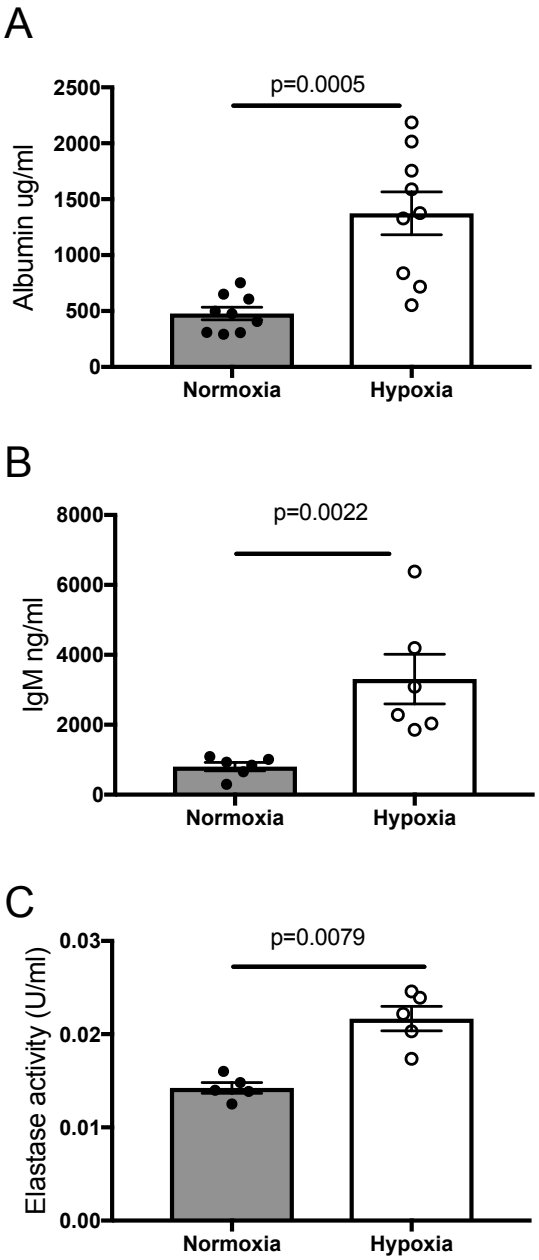


Figure 3.2: Hypoxia results in increased lung damage and increased neutrophilic inflammation

BAL supernatant from normoxic and hypoxic mice, 24 hours post-LPS was analysed for (A) albumin (n=9 over 3 experiments) and (B) IgM (n=6 over 2 experiments) by ELISA and for (C) elastase activity by the EnzCheck activity assay (n=5). Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

Hypoxia influences activation status and chemokine receptor expression in BAL neutrophils

In order to investigate the phenotypic differences between hypoxic and normoxic neutrophils, I analysed the surface receptor expression profiles of blood and BAL neutrophils from hypoxic and normoxic animals subjected to LPS induced ALI. Analysis of *ex vivo* BAL neutrophils by flow cytometry revealed evidence of increased activation with hypoxic neutrophils showing significantly increased CD11b expression (Fig 3.3A) and significantly enhanced CD62L shedding (Fig 3.3B). The chemokine receptor CXCR2 which recognises the chemokines KC (CXCL1) and MIP2 (CXCL2) mediates neutrophil recruitment to the lung[129,146,147]. Expression of this receptor on peripheral blood neutrophils is regulated by *in vivo* hypoxia with significantly lower levels of expression found on blood neutrophils from hypoxic mice (Fig 3.3C). These data suggest that other factors must play a role in neutrophil recruitment in hypoxia because the lower numbers of circulating neutrophils coupled with the preserved numbers of lung neutrophils seen in hypoxia (Fig 3.1B-D) would suggest an enhanced capacity for neutrophil recruitment in the hypoxic lung. This observation therefore requires further investigation.

Figure 3.3

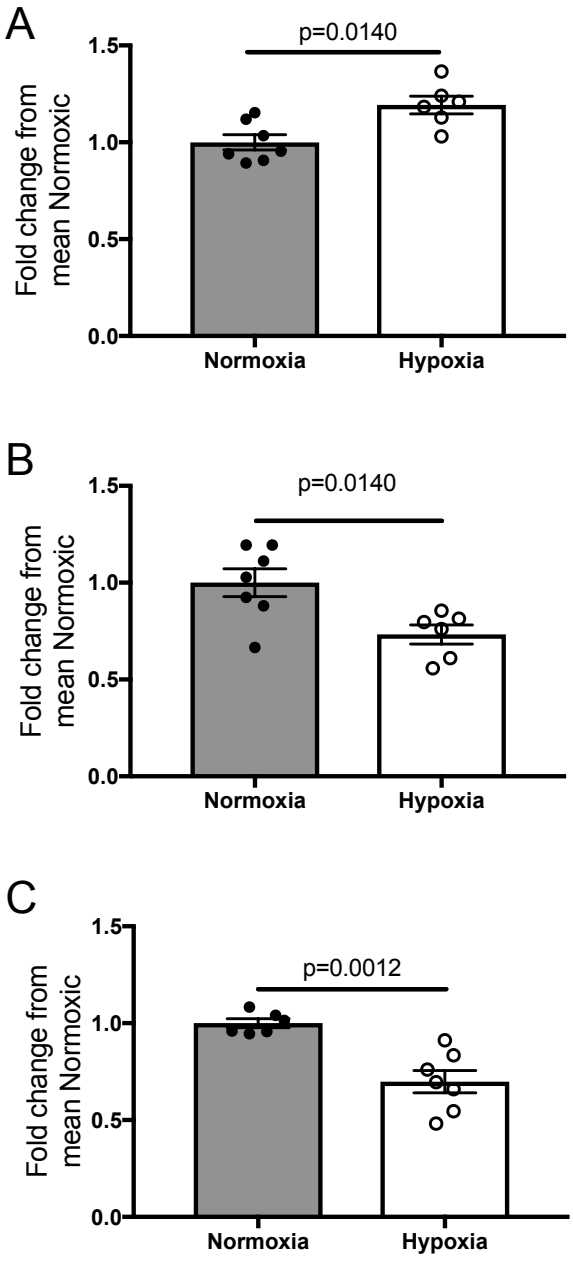


Figure 3.3: Hypoxia results in increased neutrophil activation and augmented chemokine and chemokine receptor expression
BAL neutrophils isolated 24 hours post- nebulised LPS from normoxic or hypoxic mice were analysed by flow cytometry for surface expression of (A) CD11b and (B) CD62L, n=6-7 over 2 experiments. Peripheral blood neutrophils from normoxic and hypoxic mice, 24 hours post-LPS, were analysed for CXCR2 expression by flow cytometry (C), n=6-7 over 2 experiments. (A)-(C) Mann Whitney test of significance. Data represents individual values and mean ± SEM.

Defining the proteome of inflammatory lung neutrophils

Understanding the identity of an inflammatory neutrophil is essential in devising novel therapeutic strategies to target neutrophilic inflammation. In order to characterise inflammatory lung neutrophils more fully and to determine how it is influenced by hypoxia, I undertook proteomic analysis of inflammatory lung neutrophils.

Highly pure BAL neutrophils were isolated from mice 24 hours after LPS induced acute lung injury. High resolution mass spectrometry was used to define the proteome of these cells. Three biological replicates were analysed with strong correlation between replicates (Fig 3.4A). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013672.

I identified over 5000 proteins from 57 000 peptides. The absolute copy number of proteins was calculated using the 'proteomic ruler' approach[148]. Of the ~5000 proteins, a small number account for a large proportion of the protein mass with only 17 proteins accounting for 25% of the protein mass and 73 proteins making up 50% of the protein mass (Fig 3.4B). All proteins were divided into quartiles and the top 2 quartiles were analysed for KEGG pathway enrichment (as compared to the whole neutrophil proteome). This analysis of the most abundant proteins provides insights into the key processes undertaken by inflammatory neutrophils with metabolic, migratory and inflammatory pathways being highlighted (Fig 3.4C). The 25 most abundant proteins are expanded in figure 3.4C and, again, shows the high abundance of inflammatory and metabolic proteins. I also investigated the cellular identity

of the inflammatory neutrophil by comparing the genes corresponding to the whole neutrophil proteome with the whole mouse genome. In this analysis neutrophils demonstrate enrichment in inflammatory but also metabolic pathways (Table 1 and 2). The abundant glycolytic capacity of these cells is illustrated by the very high copy number of all of the glycolytic enzymes (Fig 3.4D) with the majority of enzymes being present at >100,000 copies per cell. Despite being harvested at a time point where significant degranulation has occurred, the copy number of granule proteins from all three granule subtypes remains high (Fig 3.4E), over $1x10^6$ copies of many granule proteins were found. Both glycolytic enzymes and granule proteins are represented in the top 25 proteins (highlighted in Fig 3.4C in orange and purple respectively), along with structural proteins such as actin and histones.

Figure 3.4

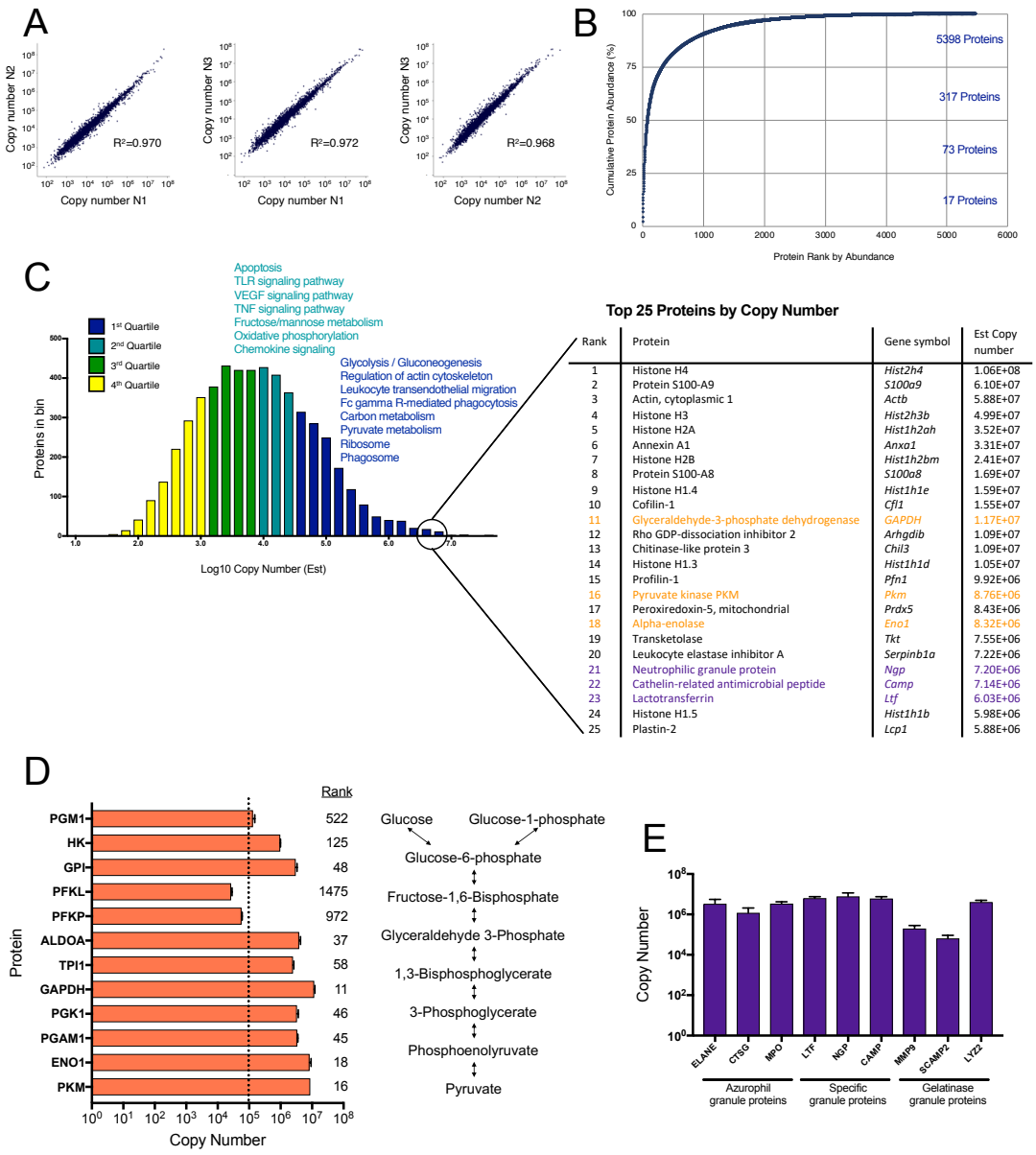


Figure 3.4: The proteome of the inflammatory neutrophil

(A) Correlation of protein copy number for three biological replicates (N1-N3) with R^2 value shown for each comparison. (B) The contribution of each protein to the total protein mass was calculated using the copy number and molecular mass for each of the 5398 identified proteins, the number of proteins accounting for percentage of total protein mass is shown in blue. (C) Proteins were divided into quartiles based on the copy number abundance and KEGG pathway analysis of enriched pathways in the top two quartiles of the proteome was carried out. Pathways enriched in the top quartile are in dark blue and those enriched in the 2nd quartile are in turquoise. The top 25 proteins (by copy number) are expanded, highlighting glycolytic enzymes (orange) and inflammatory pathways (purple). (D) Glycolytic enzymes are expressed in high copy numbers; the dotted line illustrates that most are present at more than 10^5 copies per cell. (E) Copy number of granule proteins from all granule types. For all data n=3. (B) and (C) copy number analysis based on mean of three biological replicates. (D)&(E) Data represents mean ± SEM

Table 1

KEGG Pathway Term	Count	P Value	Fold Enrichment
Fc gamma R-mediated phagocytosis	65	1.1E-13	2.118
Bacterial invasion of epithelial cells	56	9.4E-10	1.965
Lysosome	87	1.7E-14	1.952
NOD-like receptor signalling pathway	39	1.8E-06	1.906
VEGF signalling pathway	41	1.9E-06	1.870
Endocytosis	185	1.2E-24	1.821
Phagosome	105	2.6E-10	1.651
TNF signalling pathway	63	1.1E-05	1.582
Toll-like receptor signalling pathway	56	1.7E-04	1.517
HIF-1 signalling pathway	57	2.2E-04	1.500
NF-kappa B signalling pathway	49	5.8E-03	1.382
Antigen processing and presentation	41	1.5E-02	1.368
MAPK signalling pathway	124	4.2E-05	1.341
Chemokine signalling pathway	89	9.0E-03	1.243
Focal adhesion	90	3.1E-02	1.190

Table 2

KEGG Pathway Term	Count	P Value	Fold Enrichment
Pentose phosphate pathway	27	1.9E-08	2.463
Amino sugar and nucleotide sugar metabolism	44	1.3E-13	2.457
Fructose and mannose metabolism	29	5.3E-08	2.334
Citrate cycle (TCA cycle)	27	2.7E-07	2.309
Galactose metabolism	25	1.0E-05	2.138
Pentose and glucuronate interconversions	28	2.8E-06	2.128
Propanoate metabolism	21	7.9E-05	2.128
Carbon metabolism	90	5.7E-19	2.123
mTOR signalling pathway	45	2.7E-09	2.087
Biosynthesis of amino acids	56	9.4E-10	1.965
Pyruvate metabolism	28	3.3E-05	1.965
Fatty acid metabolism	35	1.1E-05	1.878
Insulin signalling pathway	90	5.2E-11	1.759
Glutathione metabolism	35	1.2E-04	1.741
Biosynthesis of unsaturated fatty acids	17	1.3E-02	1.723
Glucagon signalling pathway	62	4.9E-07	1.697
Cysteine and methionine metabolism	24	5.4E-03	1.642
Starch and sucrose metabolism	19	1.8E-02	1.625
AMPK signalling pathway	75	4.2E-07	1.616
Oxidative phosphorylation	76	1.8E-05	1.496

Tables 1 and 2: Neutrophils show enrichment for inflammatory and metabolic pathways
The genes encoding the 5398 proteins identified in the neutrophil proteome were compared with the whole mouse genome using DAVID software to identify pathway enrichment. Enriched KEGG pathway terms with their corresponding gene counts, significance and fold enrichment are shown. Table one shows selected inflammatory pathways and table 2 shows selected metabolic pathways. Raw data detailing of all the enriched pathways are in Appendix 9.

Hypoxia results in a distinct neutrophil proteomic signature

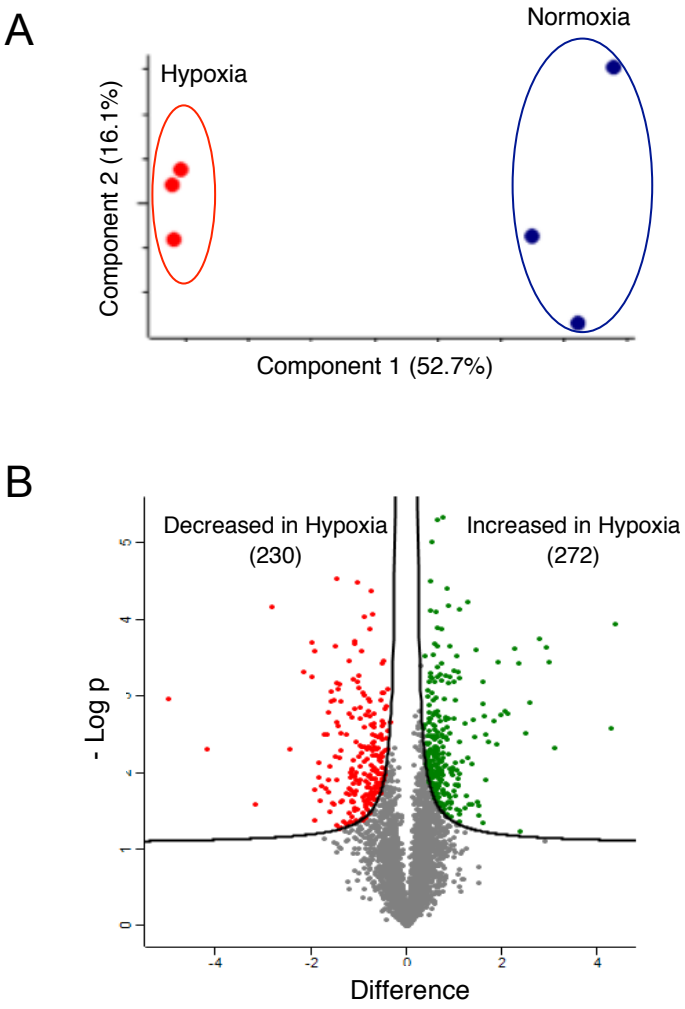
The increased neutrophilic inflammation and lung damage which is associated with hypoxia was not explained by enhanced neutrophil recruitment in hypoxic mice (Fig 3.1). I therefore hypothesised that exposure to hypoxia results in a phenotypic shift to a hyperinflammatory neutrophil causing excess tissue damage. In order to characterise this phenotype, I carried out proteomic analysis of BAL neutrophils from C57Bl6 mice which had been housed in hypoxia for 24 hours following administration of nebulised LPS. Comparison of neutrophils from hypoxic mice with those from normoxic controls confirms a distinct hypoxic proteomic signature with principle component analysis demonstrating differential clustering of the 2 groups (Fig 3.5A). The data was analysed using Perseus software[115] and this demonstrated significant upregulation of 272 proteins in hypoxic samples and down regulation of 230 proteins ($P<0.05$, FDR 0.05, $S_0=0.1$)(Fig 3.5B). KEGG pathway enrichment analysis was carried out on the significantly upregulated proteins, this highlighted four pathways which were enriched in the protein set (Fig 3.5C) including the lysosome whose relevance will be discussed in detail in the next chapter. The complement and coagulation cascade was also an interesting hit with the potential to influence inflammatory responses. More detailed analysis however identified that of the 10 pathway members which were significantly upregulated in hypoxia, four corresponded to subunits of SerpinA1 (alpha 1-antitrypsin) and three corresponded to other Serpin family members (C1, G1 and F2) which would be predicted to dampen rather than exacerbate inflammation. The complement receptor C4b was also significantly

upregulated although other complement cascade members were not regulated by hypoxia.

Overall though the pathway analysis did not identify factors which would be expected to mediate inflammation.

Taken together, these data confirmed our hypothesis that hypoxia profoundly alters the identity of a neutrophil, but the pathway enrichment analysis did not prove particularly illuminating in unpicking the mechanisms which drive the hyperinflammatory phenotype seen in hypoxia. I therefore proceeded to examine the proteome for individual proteins which were significantly upregulated by hypoxia to try to identify relevant pathways.

Figure 3.5



C

Pathways enriched in upregulated proteins

KEGG Pathway Term	Fold Enrichment	P Value
Glycosaminoglycan degradation	3.374	0.021209
Vasopressin-regulated water reabsorption	2.062	0.056719
Complement and coagulation cascades	1.996	0.050194
Lysosome	1.564	0.032311

Figure 3.5: *In vivo* hypoxia results in a distinct neutrophil proteomic signature
Proteomic analysis of BAL neutrophils from normoxic and hypoxic mice, 24 hours post LPS (n=3) were analysed using Perseus software and (A) principle component analysis was performed without category enrichment in components. (B) Volcano plot illustrating significant up (green) and downregulation (red) of over 200 proteins in hypoxic samples ($P<0.05$, FDR 0.05, $S_0=0.1$). (C) Enrichment analysis identifies KEGG pathways which are enriched in the upregulated proteins.

Hypoxia results in a hyperinflammatory phenotype with increased expression of inflammatory receptors

Analysis of those proteins which were most upregulated by hypoxia provided important insights into the behaviour of hypoxic neutrophils. In keeping with the *in vivo* phenotype of increased sickness and lung damage, I identified upregulation of a number of key inflammatory pathways in the neutrophils of hypoxic mice.

TNF-α has been shown to regulate neutrophil functions including adhesion[149], priming[47] and apoptosis[150]. I found a significant increase in TNF receptor 1B (TNFRSF1B) protein in hypoxic neutrophils(Fig 3.6A) and higher levels of TNF-α in the BAL supernatant of hypoxic mice (Fig 3.6B). I confirmed surface expression of this receptor using flow cytometry and furthermore demonstrated temporal regulation of TNF receptors: BAL neutrophils isolated 6 hours post-LPS demonstrate low levels of TNFRSF1B surface expression, however, by 24 hours the surface expression has increased, and significantly higher levels are seen in hypoxic compared with normoxic samples (Fig 3.6C). This pattern of expression was specific to TNFRSF1B as demonstrated by the distinct expression pattern of TNFRSF1A (Fig 3.6D) which showed significantly higher expression of in hypoxia at 6 hours but no difference in expression between normoxic and hypoxic mice at 24 hours. The TNFRSF1A protein was not identified in the proteomic dataset. Furthermore, the regulation of TNF receptor expression was specific to BAL neutrophils. Flow cytometric analysis of peripheral blood neutrophils at 6 and 24 hours post-LPS showed no evidence of hypoxic regulation of either

TNFRSF1B or TNFRSF1A expression at either time point (Fig 3.7A&B). I noted variation in the raw fluorescence values between experiments and so, in order to avoid missing a signal, I replotted the data for blood TNFRSF1B expression at 24 hours as a fold change and reanalysed (Fig 3.7C), this showed a trend towards increased expression in hypoxia but did not meet statistical significance.

The effects of TNF-α on neutrophil functions are complex, varying over the time course of an inflammatory response. Additionally, the two different receptors are thought to have different downstream effects. The data described here suggests that temporal regulation of receptor expression may play a role in determining TNF-α responsiveness in inflammatory neutrophils and that hypoxia may further regulate the expression of these receptors.

Formylated peptide receptors are G-protein-coupled receptors (GPCRs) whose ligands include bacterial and mitochondrial formylated peptides. Upon activation, FPRs induce intracellular signalling cascades which result in neutrophil chemotaxis, degranulation, ROS production and transcriptional regulation[12]. Both FPR1 and FPR2 were significantly upregulated in the proteome of inflammatory neutrophils (Fig 3.8A), consistent with the hyperinflammatory phenotype.

Figure 3.6

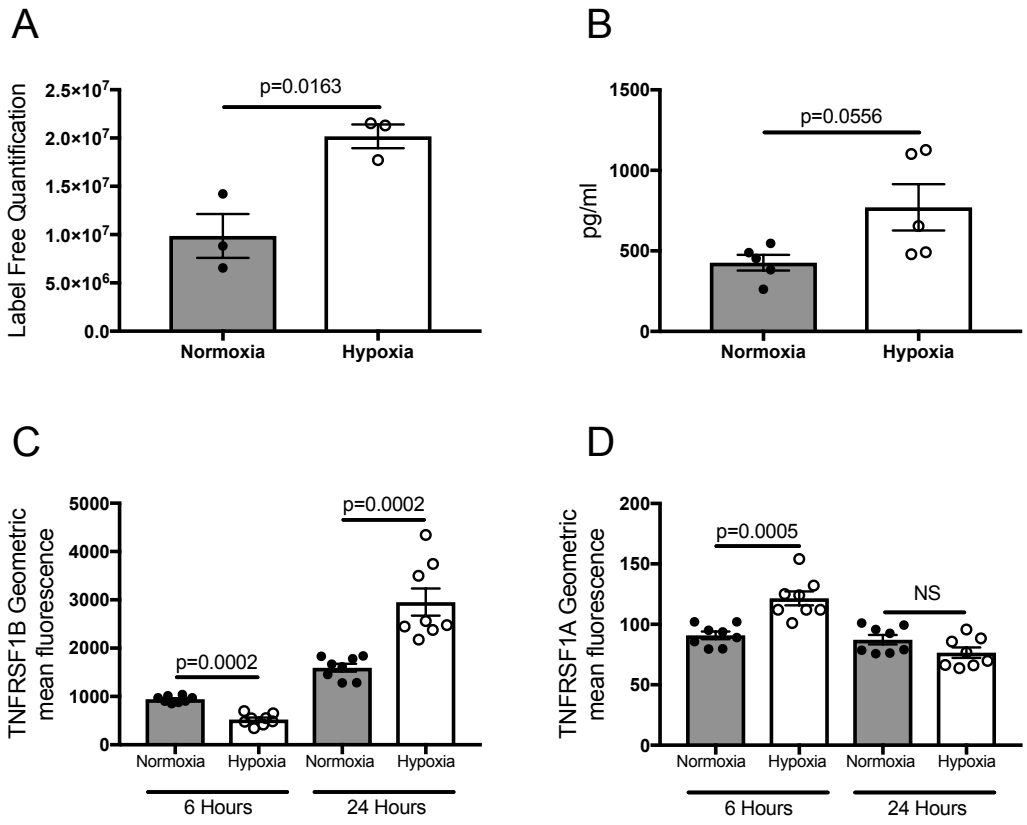


Figure 3.6: BAL neutrophil TNF receptor expression is modulated by *in vivo* hypoxia
(A) Label free quantification of TNFRSF 1B protein expression in BAL neutrophils from normoxic and hypoxic mice, 24 hours post-nebulised LPS, n=3. (B) BAL supernatant TNF-α concentration measured by multiplex cytokine assay, n=5. Flow cytometry was used to analyse surface expression of (C) TNFRSF1B and (D) TNFRSF1A on BAL neutrophils, from normoxic and hypoxic mice, 6 and 24 hours post-LPS, n=8 over 2 experiments. (A) analysed by unpaired two tailed t-test (B)-(D) Mann-Whitney test of significance. Data represents individual values and mean $\pm$ SEM.

Figure 3.7

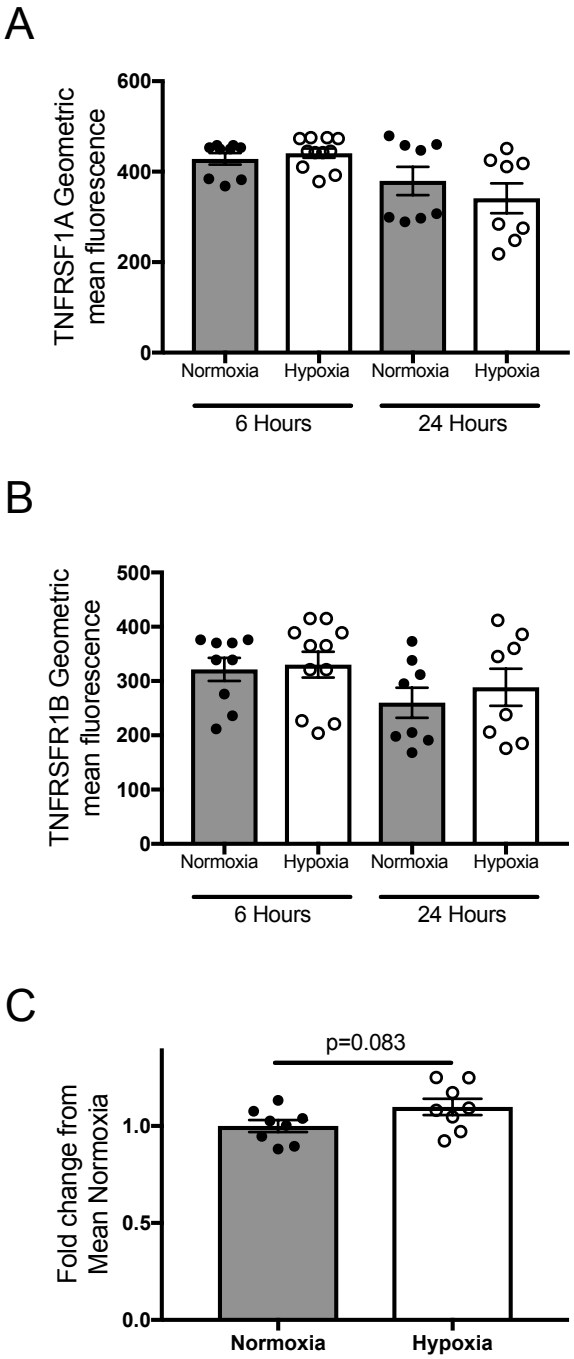


Figure 3.7: Hypoxia does not significantly alter blood neutrophil TNF receptor expression
Peripheral blood neutrophils from normoxic and hypoxic mice, 24 house post-nebulised LPS were analysed by flow cytometry for surface expression of (A) TNFRSF1A and (B) TNFRSF1B. (C) 24-hour TNFRSF1B blood neutrophil expression expressed as fold change. (A)-(C) n≥8 over 2 experiments, Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

Figure 3.8

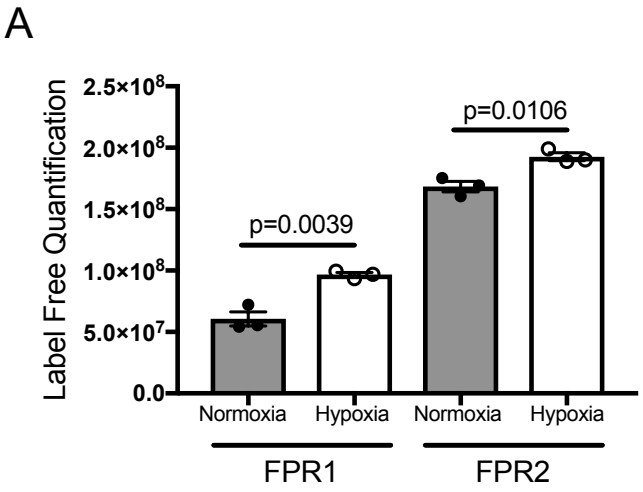


Figure 3.8: Formylated peptide receptors are upregulated in hypoxia
(A) Label free quantification of FPR1 and FPR2 protein expression in BAL neutrophils from normoxic and hypoxic mice, n=3, unpaired two tailed t-test. Data represents individual values and mean ± SEM.

Hypoxia increases GM-CSF receptor expression in neutrophils

Like TNF-α, GM-CSF is an important regulator of multiple neutrophil functions including activation, degranulation and survival. The functional GM-CSF receptor is composed of a heterodimer consisting of an alpha and a beta subunit. The beta subunit of the GM-CSF receptor (CSF2RB) was one of the most upregulated proteins in hypoxia with a 7-fold increase in label free quantification (Fig 3.9A). Surface expression of the GM-CSF receptor on neutrophils in the lungs would be predicted to contribute to increased release of granule proteins and therefore increased tissue injury. In order to investigate the surface expression of this receptor I carried out flow cytometry analysis of freshly isolated BAL neutrophils. This identified significantly higher surface expression of CSF2RB in hypoxic neutrophils 6 hours post-LPS (Fig 3.9B), as well as significantly higher levels of the co-receptor CSF2RA at this time point (Fig 3.9C). The trends towards increased hypoxic neutrophil CSF2RB expression persists until 24 hours but is not statistically significant. The protein quantification noted in Fig 3.9A will represent both surface receptor and internalized protein. I hypothesised that the high protein level seen at 24 hours is therefore the result of high levels of receptor activation and internalisation between 6 and 24 hours. I then investigated the expression of GM-CSF in the BAL fluid over the course of the inflammatory response. This was consistent with GM-CSF being an early signal in acute lung injury, showing that GM-CSF concentrations at 2 hours were approximately 100-fold higher than at 24 hours. It was interesting to find that at each time point GM-CSF levels appeared lower in hypoxic BAL supernatant although whether, at such high concentrations,

this difference is rate limiting is difficult to say. Upregulation of GM-CSF receptor expression early in the inflammatory response is important in the context of work by Hoenderdos et al[142] which demonstrated that hypoxia results in enhanced neutrophil degranulation. Consistent with this hypothesis, our proteomic data identifies significant upregulation of the downstream regulator of GM-CSF mediated degranulation, Rab27a[151] in hypoxic neutrophils (Fig 3.9E).

Figure 3.9

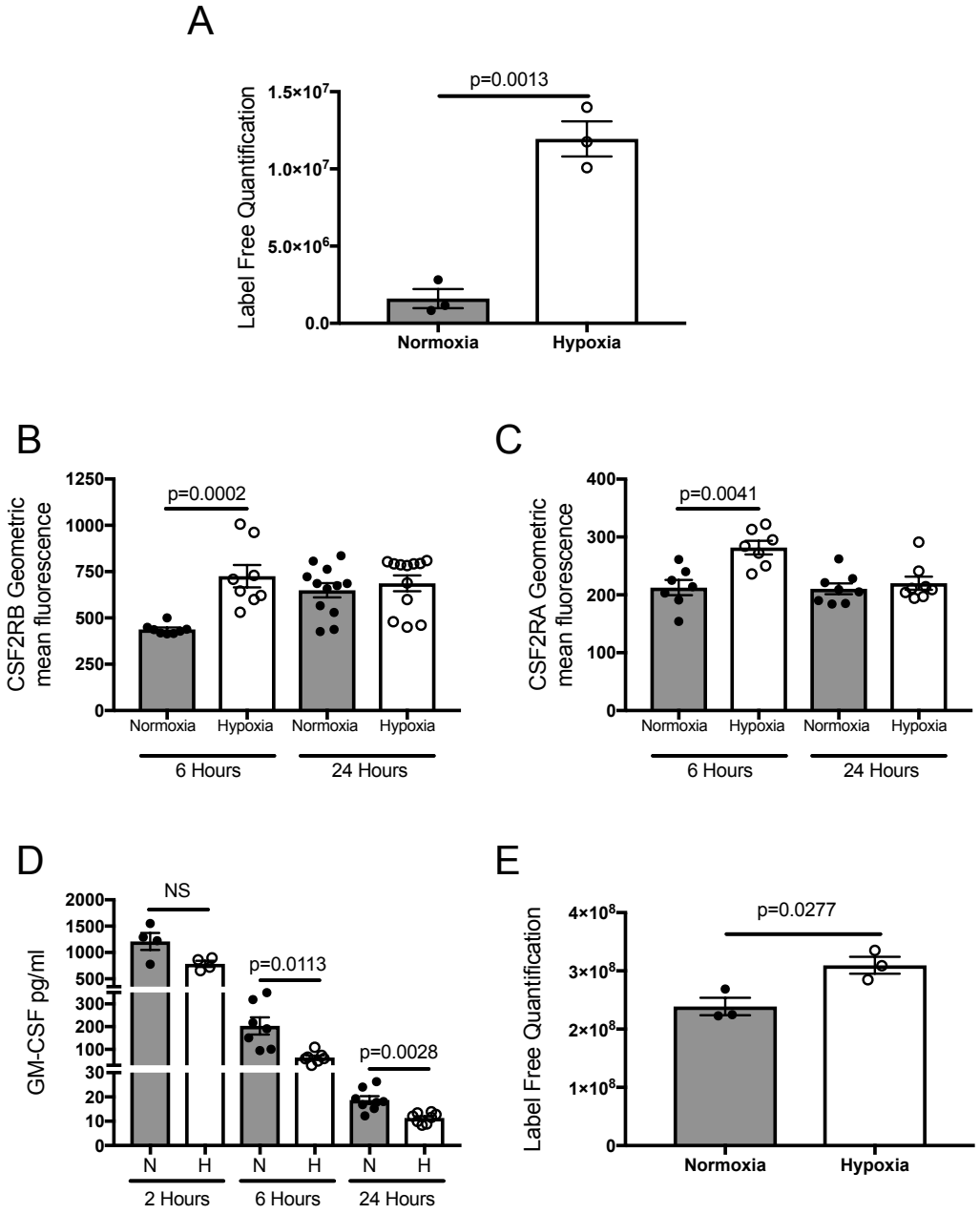


Figure 3.9: The GM-CSF pathway is upregulated in hypoxic neutrophils
(A) Label free quantification of CSF2RB protein expression in BAL neutrophils from normoxic and hypoxic mice, n=3. Surface expression of (B) CSF2RB and (C) CSF2RA on BAL neutrophils, from normoxic and hypoxic mice, 6 and 24 hours post-nebulised LPS, n≥7 over 2-3 experiments. (D) Normoxic and hypoxic mouse BAL supernatant GM-CSF concentration, measured by ELISA at 2, 6 and 24 hours post-nebulised LPS n≥4. (E) Label free quantification of Rab27A protein expression in BAL neutrophils from normoxic and hypoxic mice, n=3, unpaired two tailed t-test. (A)&(E) unpaired two tailed t-test, (B)&(C) Mann-Whitney test of significance (D) significance analysed by multiple t-tests (corrected for multiple comparisons using Bonferroni method). Data represents individual values and mean ± SEM.

Hypoxia results in enhanced neutrophil degranulation *in vivo*

I went on to investigate whether increased GM-CSF receptor expression at the earlier, 6-hour, time point was associated with *in vivo* evidence of altered degranulation in hypoxia. I found that 6 hours post-LPS there is significantly greater neutrophil elastase activity (Fig 3.10A) and MMP9 (Fig 3.10B) in the BAL supernatant and a trend towards increased myeloperoxidase (MPO) activity (Fig 3.10C). This early increase in degranulation is associated with significantly increased lung injury at this time point in hypoxia with increased albumin leak at 6 hours (Fig 3.10D). There are fewer neutrophils in the BAL fluid of hypoxic mice at 6 hours compared with normoxic mice (as described in Fig 3.1B), suggesting an even greater propensity to degranulate than the BAL supernatant data alone demonstrates. These findings suggest that early enhanced degranulation may be a contributing factor to the increased lung damage seen in hypoxia.

Figure 3.10

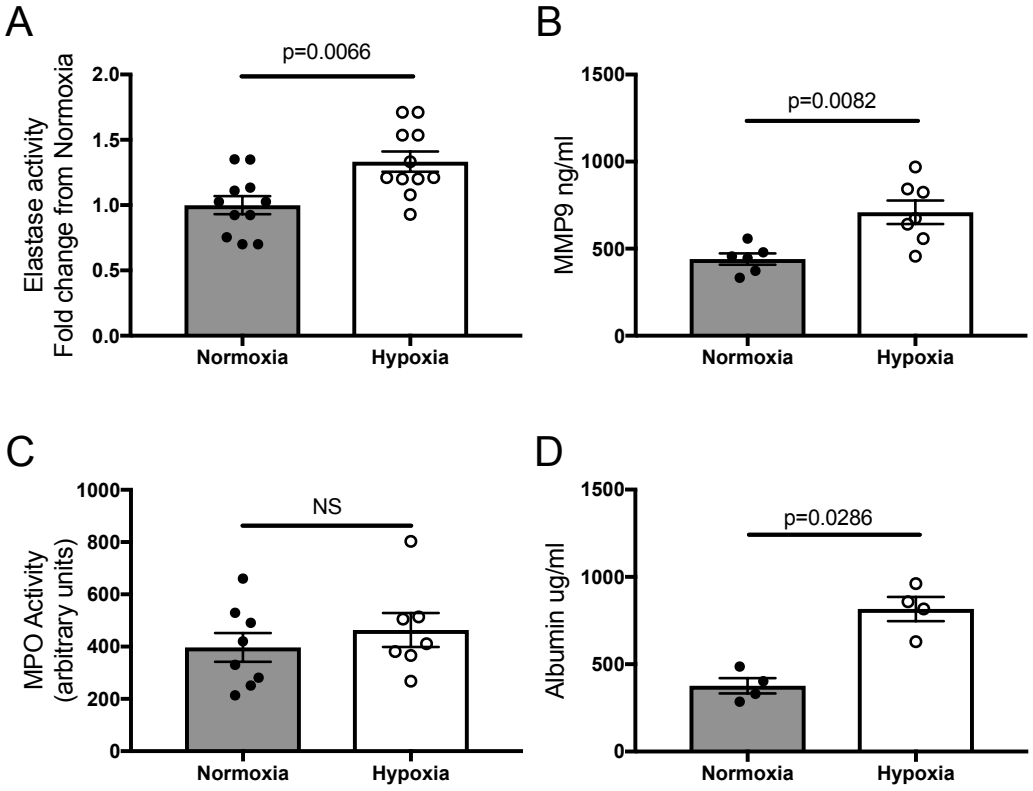


Figure 3.10: *In vivo* hypoxia leads to increased neutrophil degranulation at 6 hours
BAL supernatant was collected from mice 6 hours post-LPS and analysed for (A) elastase activity, (B) MMP9 concentration, (C)MPO activity and (D) albumin concentration. Elastase and MPO activity were measured by EnzCheck activity kits and MMP9 and albumin concentration analysed by ELISA. (A)-(C) n≥6 over 2-3 experiments, (D) n=4. All data analysed by Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

Hypoxic neutrophils show preserved intracellular granule protein expression

Despite strong evidence of enhanced degranulation in hypoxia, at a protein level there was no corresponding decrease in these intracellular granule proteins at 24 hours when comparing hypoxic to normoxic samples (Fig 3.11A-C). Analysis by western blot supported this finding (Fig 3.11D-F). This is consistent with the data in Fig 3.4E showing high copy numbers of many granule proteins at the 24-hour time point, despite significant degranulation occurring by this time point.

Taken together these data show that, in the context of lung inflammation, hypoxia represents a damaging 'second hit' associated with significant hypothermia and lung damage. I have also shown evidence of increased neutrophilic activation in hypoxic animals. Inflammatory neutrophils dynamically regulate expression of inflammatory receptors and this too is modulated by exposure to hypoxia. Persistence of granule proteins in hypoxic neutrophils, despite increased degranulation, raised the interesting possibility that neutrophils may continue to synthesise inflammatory mediators upon recruitment to the hypoxic inflammatory niche. TaqMan analysis of gene expression confirmed that BAL neutrophils possess mRNA for granule proteins in inflammatory airway neutrophils (Figure 3.12 A-C) although levels are not upregulated in hypoxic neutrophils.

Figure 3.11

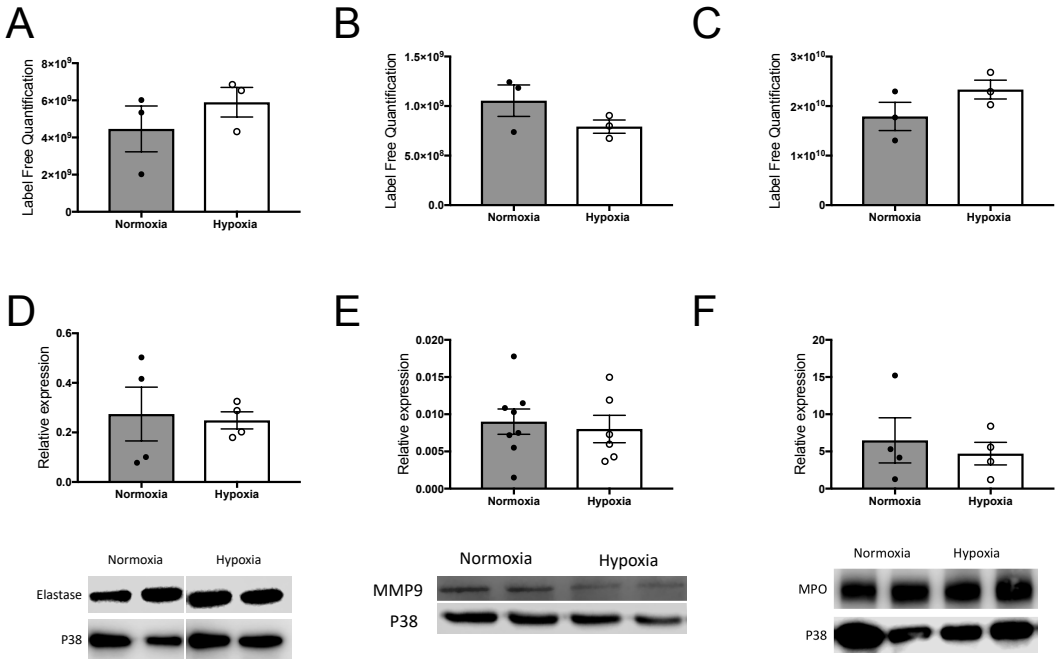


Figure 3.11: Enhanced hypoxic degranulation does not result in a reciprocal drop in intracellular granule proteins
Label free quantification of (A) elastase, (B) MMP9 and (C) MPO protein expression in BAL neutrophils from normoxic and hypoxic mice, n=3. Protein expression by western blot of (D) elastase, (E) MMP9 and (F) MPO in BAL neutrophils from normoxic and hypoxic mice 24 hours post-LPS. n≥4 over 2 experiments, representative blot shown for each protein with p38 loading control. (A)-(C) unpaired two tailed t-test, (D)-(E) Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

Figure 3.12

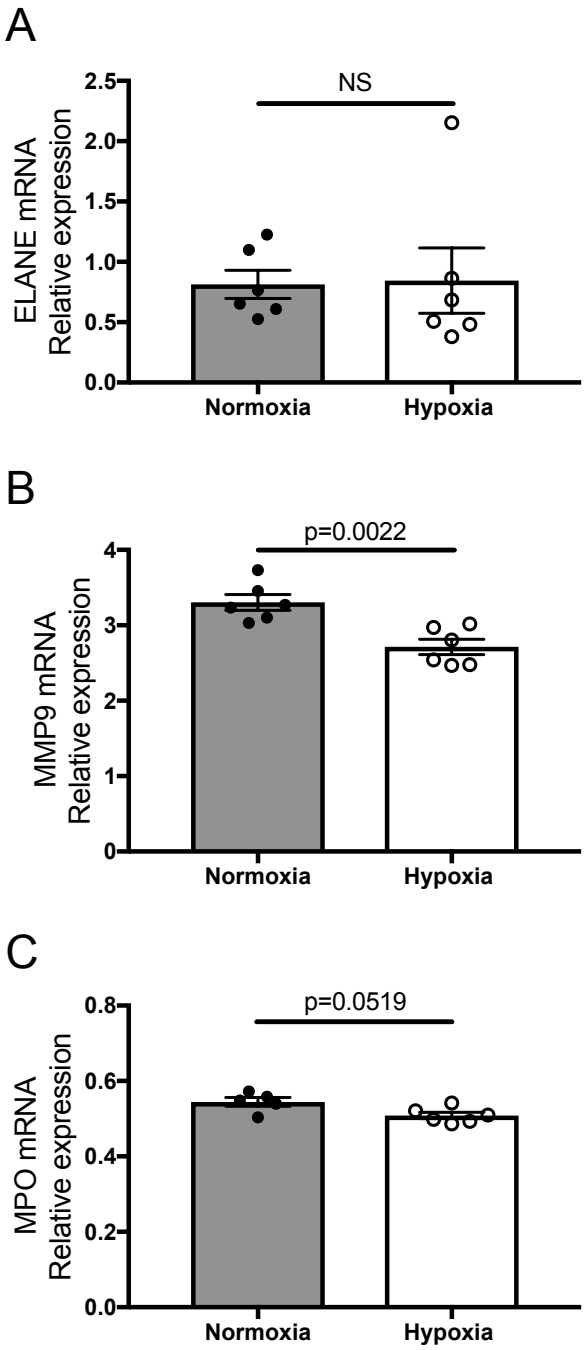


Figure 3.12: BAL neutrophils continue to express transcript for granule proteins
(A)-(C) Expression of granule content genes was analysed by TaqMan analysis of highly pure BAL neutrophils from mice 24 hours post-LPS which had been housed in either normoxia or hypoxia. Data is expression relative to beta-actin gene expression. n≥5, Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

Inflammatory lung neutrophils synthesise granule proteins and inflammatory mediators

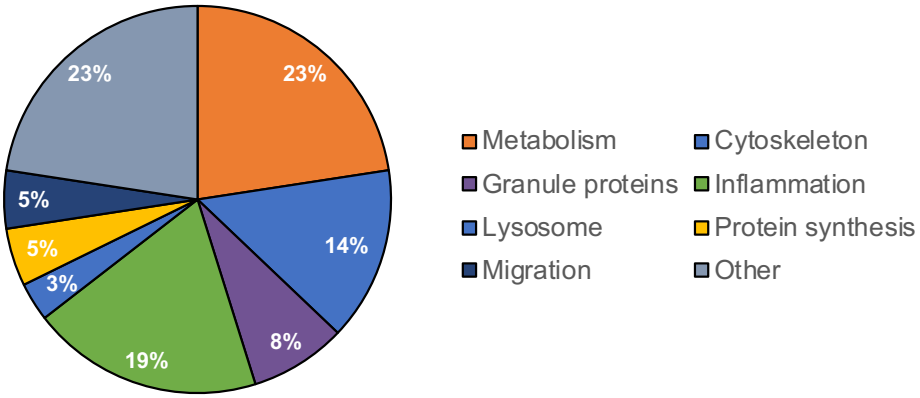
In order to investigate the synthetic capacity of inflammatory BAL neutrophils I utilised an alternative proteomic approach. By incubating *ex vivo* BAL neutrophils with the heavy labelled amino acids $^{13}C_6$ and $^{15}N_4$ L-arginine (R10) and $^{13}C_6$ L-Lysine (K6) I was able to identify newly synthesised proteins by mass spectrometry: only proteins which were newly synthesised would contain a mass shift associated with incorporation of a labelled amino acid. I used BAL neutrophils from animals which had been housed in hypoxia following LPS stimulation because I hypothesised that these would have the greatest capacity for generating inflammatory mediators, based on the evidence presented in this chapter. Following purification, neutrophils were cultured in hypoxia with labelled amino acids (or control media with unlabelled amino acids) for 18 hours prior to lysis and analysis by mass spectrometry. I identified 62 proteins which were labelled in at least 2 of the 4 labelled samples and demonstrated either no label or significantly less label in the control samples. These newly synthesised proteins included those involved in cytoskeletal regulation and metabolism (Fig 3.13A). However, I also identified inflammatory proteins such as complement C3 and integrins (Fig 3.13B) and, importantly, some known granule proteins including the protease cathepsin D and lysozyme C2 (Figure 3.13C).

These data provide evidence of ongoing synthesis of inflammatory mediators, including components of neutrophil granules, by tissue neutrophils. These data, coupled with the evidence of preserved intracellular granule proteins

despite enhanced degranulation, suggests that de novo synthesis of inflammatory mediators is a mechanism by which hypoxic neutrophils drive a hyperinflammatory immune response.

Figure 3.13

A



B

Inflammation	
Neutrophil cytosol factor 1	Leukotriene A-4 hydrolase
Cis-aconitate decarboxylase	Complement C3
Chitinase-3-like protein 1	Integrin beta-2
Macrophage migration inhibitory factor	Protein S100-A8
Interleukin-36 gamma	Protein S100-A9
Interleukin-16	Integrin alpha-M

C

Granule proteins
Monocyte differentiation antigen CD14
Beta-2-microglobulin
Cathepsin D
Lysozyme C-2
Neutrophilic granule protein

Figure 3.13: Inflammatory neutrophils synthesise granule proteins and inflammatory proteins
Labelled proteins (total number =62) were assigned to a functional group as detailed in (A). Those involved in inflammation (B) and neutrophils granules (C) are detailed below. The raw data is in Appendix 10.

Discussion

The data in this chapter confirm that exposure to hypoxia can dramatically alter the course of an inflammatory response, mirroring our group's previous findings in infection models [143]. Importantly, in the context of infection, the sickness associated with hypoxia was not related to bacterial burden but due to the host response. Neutrophil depletion resulted in partial recovery of the hypothermia observed in hypoxia, implicating these cells in the pathogenesis of this phenotype. However, the underlying mechanisms have not been fully resolved. In order to address this question of how hypoxia regulates neutrophil mediated inflammatory responses, I utilised a sterile injury model. I confirmed that in this model, *in vivo* hypoxia results in significant hypothermia and lung damage. Increased neutrophil elastase activity in the BAL fluid supernatant of hypoxic mice again implicates neutrophilic inflammation in the damage however, the phenotype is independent of neutrophil numbers. As discussed in the introduction, neutrophils are increasingly understood to have varying subtypes or polarisation states both in the context of tumour biology and inflammation[144,145]. Studies of circulating blood neutrophils in patients with sepsis[152], COPD[153] and ARDS[25] have also identified different neutrophil phenotypes thought to contribute to disease. The finding that the excessive neutrophilic inflammation observed in hypoxic animals with ALI is independent of neutrophil number is suggestive of an altered and proinflammatory neutrophil phenotype in this context. Consistent with this, hypoxia was associated with enhanced CD11b expression and shedding of CD62L, often described as an activated neutrophil phenotype. These data are

consistent with data from Juss et al[25] showing that in patients with acute lung injury, both blood and alveolar neutrophils show enhanced CD11b expression and CD62L expression.

Microenvironmental cues have been identified as important regulators of immune cell polarisation in both the macrophage and T cell populations and hypoxia and hypoxic signalling pathways have been identified as one such cue. Considerable interest exists in therapeutic targeting of hypoxic signalling pathways in the context of pathological inflammation. In some tissues, for example in the intestine, hypoxia, and specifically HIF-1α activity, are viewed as protective, anti-inflammatory signals[154,155]. Prolyl hydroxylase inhibitors which potentiate HIFα activity have shown promise in treating intestinal inflammation in mouse models of colitis[156-158]. There is also some evidence that HIF-1α activity in the alveolar epithelium may be protective in stretch induced acute lung injury[80]. However, in a different model, alveolar epithelial cell HIF-1α activity was implicated in dysfunctional airway remodelling after influenza infection[81]. These contrasting findings highlight the complexity and context specificity of hypoxic responses in inflammation. Hypoxia frequently complicates both acute and chronic respiratory disease. This hypoxia may be local or systemic and as such will have wide ranging effects on immune cells but also potentially on the endothelium and epithelium. The overall effect of a hypoxic challenge will therefore represent the sum of effects on multiple tissues and the impact of hypoxia on infiltrating immune cells will be an important determinant of the overall outcome of a hypoxic inflammatory response.

Ex vivo studies of neutrophils in hypoxia have proved invaluable in unravelling how these cells respond to hypoxia. However, there is an increasing awareness that multiple factors in the inflammatory microenvironment, not just hypoxia, influence neutrophil function *in vivo*. Furthermore, the process of transmigration fundamentally alters neutrophil identity. Replicating these events *in vitro* is virtually impossible and so experimental models which allow analysis of neutrophils exposed to hypoxia *in vivo* in the inflammatory environment are important in understanding how these tissue cells behave. Proteomic analysis of immune cells provides a quantitative and unbiased assessment of the identity of these cells.

Proteomic analysis of inflammatory neutrophils has provided insights into the important pathways and processes which are active in these cells. Comparison of genes encoding the neutrophil proteome with the whole mouse genome highlights enrichment of metabolic processes and inflammatory pathways. The presence of metabolic enzymes in the 'top 25' list and their predominance in the top quartiles for protein abundance further highlights their importance. It has long been established that neutrophils are primarily glycolytic cells[159] and derive little or no energy from aerobic respiration which occurs within the mitochondria. Neutrophils do possess a network of mitochondria and express at least some of the machinery of the TCA cycle essential for aerobic respiration. Our group has previously investigated the role of this machinery in neutrophils through examining the neutrophils of patients with a mutation in the TCA cycle enzyme succinate dehydrogenase B (SDHb) [160] and found that loss of this enzyme altered neutrophil metabolism, ROS production and

survival. Fossati et al[161] have investigated the role of mitochondria in neutrophils through the use of chemical inhibitors and found that intact neutrophil mitochondria were required for chemotaxis and may be required for maximal respiratory burst capacity. Given this, it was interesting to find that not only were neutrophils enriched for glycolysis and the related KEGG pathways of fructose and pyruvate metabolism, but they were also enriched for TCA cycle proteins. Additionally, neutrophils were enriched for pathways involved in fatty acid metabolism. Fatty acid metabolism in neutrophils has recently been linked with differentiation of bone marrow neutrophils[162] but its role in inflammatory neutrophils has not been investigated. Our proteomic data shows a nearly 2-fold enrichment in this KEGG pathway suggesting it may have important roles in inflammatory neutrophils as well as in differentiating bone marrow neutrophils.

Of particular interest in the enrichment analysis are those pathways which link inflammation, hypoxia and metabolism such as the HIF, mTORC and AMPK signalling pathways. Relatively little is known about mTOR and AMPK signalling in neutrophils. These pathways and the role of metabolic adaptations to hypoxia will be discussed in more detail in the next chapter.

Comparison of the proteome of normoxic and hypoxic neutrophils confirms that hypoxia significantly alters neutrophil phenotype. Hypoxia resulted in significant up- and down-regulation of hundreds of proteins. Pathway enrichment analysis did not at first identify clear links between the altered neutrophil proteome and the hyperinflammatory *in vivo* phenotype. The identification of the lysosome as a pathway enriched in the upregulated

proteins is of interest and will be discussed in detail in the following chapter. Analysis of those proteins most significantly upregulated by hypoxia highlighted that hypoxic neutrophils upregulate a range of inflammatory receptors including TNF receptor 1b, formylated peptide receptors and the GM-CSF receptor. The repertoire of receptors expressed on the neutrophil cell surface has important implications for pathogen recognition, effector functions such as chemotaxis and phagocytosis and neutrophil survival. Surface expression of a given receptor may be regulated at multiple levels: transcription and translation of receptor protein, post-translational modifications and trafficking of the receptor to the cell surface. Additionally, many receptors require cofactors or coreceptors to function at the cell surface and so availability of these will further regulate receptor activity. In neutrophils, degranulation may also alter surface receptor expression. TNF receptors and formylated peptide receptors are both expressed in the inner membrane of specific granules in neutrophils and so, upon fusion of the granule membrane with the plasma membrane, these receptors may be presented on the cell surface. Analysis of TNF receptor expression by flow cytometry provided further insights into how this pathway is regulated. I found that surface expression of TNF receptors changes over the time course of an inflammatory response in the lung. This was particularly true for TNFRSF1B which is lower in hypoxic samples at 6 hours but dramatically higher in hypoxic samples by 24 hours. Comparison of the 6-hour BAL neutrophil TNF receptor data with the data from 24 hours suggests that surface expression of the TNF receptor 1B increases between 6 and 24 hours. Technical factors and experimental design

mean that it is difficult to draw absolute conclusions from this flow cytometry data from different time points but the experimental protocol, antibody concentration and flow cytometer settings were unchanged between experiments, so it is likely that TNFRSF1B surface expression increases over time. This finding would be consistent with increased degranulation over the time course of the inflammatory response although TNFRSF1A does not demonstrate the same changes in surface expression over time, suggesting that it is either not present in granules or is also regulated by other factors.

Flow cytometry was also important in delineating the time course of GM-CSF receptor expression in BAL neutrophils. These data showed early hypoxic upregulation of the GM-CSF receptor at a surface level. While the proteomic data shows a persistent increase in the beta subunit in hypoxic samples this is not reflected in the flow cytometry data. Ligation and activation of the GM-CSF receptor on neutrophils leads to its internalisation by endocytosis. Interestingly, stimulation of neutrophils with both TNF-α and fMLF also led to downregulation of the GM-CSF receptor at the cell surface[163] which may explain the high protein but low surface expression seen in hypoxic neutrophils by 24 hours. Although levels of formylated peptides were not measured in the BAL supernatants in this experimental model, levels of mitochondrial formylated peptides have been shown to be increased in both the serum and BAL fluid of patients with ARDS, highlighting their role in this disease[164].

Hypoxia has been found to regulate neutrophil degranulation *in vitro* in response to a range of stimuli, including GM-CSF[142]. The data in this chapter confirm that *in vivo* hypoxia regulates neutrophil degranulation and

that this occurs early in the course of the inflammatory response. Furthermore, early degranulation leads to early lung damage in hypoxic mice. The lack of a reciprocal reduction in hypoxic neutrophil intracellular granule proteins led to the hypothesis that hypoxic neutrophils may continue to synthesise these inflammatory mediators in situ. This would clearly go against the paradigm that granule proteins are only synthesised during neutrophil maturation in the bone marrow. TaqMan analysis of BAL neutrophil gene expression confirms the presence of elastase, MMP9 and MPO transcripts. Hypoxia did not upregulate the abundance of these transcripts in BAL neutrophils from the 24-hour time point. This suggests that expression of these proteins may be regulated at a translational or post-translation level.

By culturing neutrophils in the presence of labelled amino acids, I was able to delineate a subset of proteins which are synthesised de novo by tissue neutrophils. The identification of newly synthesised inflammatory mediators and granule proteins is an important finding as it identifies a novel mechanism by which neutrophils, given the right stimuli, continue to cause tissue damage following transmigration. The data in this chapter suggest that hypoxic neutrophils display a greater capacity for the synthesis of granule proteins, based on their increased degranulation without reduced intracellular granule protein content. One mechanism by which hypoxic neutrophils might upregulate expression of these inflammatory mediators is through increased substrate availability and this hypothesis will be addressed in the next chapter. The data in this chapter also suggest that, in hypoxia, there may be failure of inflammation resolution. While there are fewer neutrophils in the BAL fluid of

hypoxic mice up to 24 hours following LPS, by 48 hours there is a trend towards more neutrophils in hypoxic mice. This is in keeping with persistence of neutrophils in hypoxic lungs. Multiple factors are likely to contribute to this persistent inflammation. Hypoxia itself is a profound survival stimulus for neutrophils. I have shown that hypoxia upregulates TNF receptor 1B expression and TNF signalling through the TNFRSF1B receptor may also contribute to increased neutrophil survival. The effects of TNF-α on neutrophil survival are complex and while TNF-α may be pro-apoptotic at early time points[165], but in primed neutrophils and at later time points it is pro-survival. Furthermore, TNFRSF1A, rather than TNFRSF1B, is the dominant receptor in relaying pro-apoptotic effects[150] so overall the upregulation of TNFRSF1B at the later, 24-hour time point is likely to enhance survival of neutrophils. It is also probable that hypoxic neutrophils have a degree of metabolic advantage over their normoxic counterparts. As discussed above, neutrophils express the machinery for carbohydrate, fat and protein metabolism and the role of these in hypoxic neutrophils will be addressed in the next chapter.

In summary, hypoxia drives neutrophilic inflammation in the lung through upregulation of inflammatory receptors, enhanced neutrophil degranulation and persistence of neutrophilic infiltrates. I went on to interrogate the hypoxic neutrophil proteome to understand how this enhanced inflammation is fuelled in hypoxia.

Chapter 4: Protein Scavenged from the Extracellular Environment Fuels Neutrophilic Inflammation in Hypoxia

Introduction

The data discussed in the previous chapter supports the hypothesis that hypoxia results in a hyperinflammatory neutrophil phenotype. An early feature of this phenotype is the increased GM-CSF receptor expression and degranulation which occurs. This results in significant lung damage. The enhanced degranulation without a reduction in levels of intracellular granule proteins is suggestive of continued synthesis of inflammatory mediators by hypoxic neutrophils. These data, coupled with the persistence of neutrophilic infiltrates in hypoxia would suggest a requirement for substrate, both in the form of energy and amino acids for de novo protein synthesis. As noted in figure 3.12, this program of protein synthesis is not regulated at a transcriptional level. This observation led me to consider whether hypoxic neutrophils may have an advantage in terms of substrate availability.

As discussed previously, neutrophils are highly glycolytic at baseline and generate little to no ATP through aerobic respiration, irrespective of oxygen availability meaning that they are “pre-adapted” to function in low oxygen environments. This is logical in cells which, as the first responders to infection and inflammation, are often required to function in environments where oxygen is limited. In these situations, nutrient availability may also be limited, and it is interesting to consider how the highly glycolytic neutrophil may respond and adapt to this additional metabolic stress. There are multiple parallels between

the hostile inflammatory environment in which neutrophils function and the tumour microenvironment. Solid tumours often proliferate to the extent that they out-strip their blood supply and as a consequence develop regions deficient in both oxygen and nutrients. Adaptations to these environmental stresses are often noted in tumour cells and are under investigation as potential therapeutic targets[166-168], reviewed in[169]. Another interesting link between tumour cell and neutrophil metabolism is the Warburg effect[170] which describes a switch from aerobic metabolism to glycolysis in tumour cells, even in the presence of adequate oxygen, mirroring the baseline glycolytic metabolism of neutrophils described above.

The airway has been shown to represent a relatively low glucose environment in comparison to the circulation[171]. This is in stark contrast to standard cell culture media which is rich in both glucose and amino acids such as glutamine. Thus, immune cells which have transmigrated to the airways will travel down a glucose gradient, arriving in a glucose deplete environment. It is therefore important to consider alternative sources of nutrients in this context and tumour biology may provide some clues in this regard.

Macropinocytosis is a process by which cells take up extracellular fluid and its constitutive molecules, including proteins. This non-specific uptake is a highly conserved process and has consequences for cellular motility, metabolism, and immunity[172]. Macropinocytosis has been identified as a route by which tumour cells may adapt to low nutrient availability: it is found to be upregulated in Ras -transformed pancreatic tumour cell lines and has been shown to be a key mechanism of nutrient acquisition in these cells[173-175].

Macropinocytosed proteins are trafficked to the lysosome where they are broken down to their constitutive amino acids. Glutamine from macropinocytosed proteins can be utilised for ATP generation via central carbon metabolism[173]and is required for *in vitro* tumour cell proliferation and *in vivo* tumour growth. Neutrophils have been shown to carry out macropinocytosis[176], although this process and the factors which regulate it are not well studied in these cells. In contrast, the capacity of other immune cells, particularly antigen presenting cells such as monocytes[177] and dendritic cells[178] to carry out macropinocytosis is well established and, in contrast to most tumour cells, may be constitutive as well as in response to stimulus[179,180] . Carpentier et al utilised electron microscopy to investigate the internalisation and subsequent localisation of the complement receptor CR1 in neutrophils and found that this occurs via macropinocytosis and was augmented by fMLF and PMA stimulation. This process did require anti-CR1 antibody so the capacity of neutrophils to carry out non-specific macropinocytosis and the factors regulating this remain uncertain. Nutrient acquisition by macropinocytosis has not previously been studied in neutrophils.

The uptake and breakdown of proteins by tumour cells is a highly regulated process. The mammalian target of rapamycin (mTOR) and its associated complexes, mTORC1 and mTORC2, are essential in integrating signals regarding nutrient availability and determining the cellular response to these signals in terms of transcription and translation of new proteins and in directing proliferation. Multiple environmental factors upstream of mTORC signalling

feed into this pathway, including growth factors, amino acid availability, oxygen tension and glucose availability. The downstream consequences of mTORC1 and mTORC2 activation are varied. mTORC1 primarily directs the synthesis of proteins, lipids and nucleotides, required for cell growth while mTORC2 regulates cell survival and proliferation[91]. Consistent with this, Palm et al[174] have demonstrated that, in Ras transformed pancreatic cancer cells, mTORC1 plays an essential role in regulating protein catabolism in response to amino acid starvation. The authors show that when free amino acids are in abundance, mTORC activity promotes cells growth, however, in low amino acid conditions, when the cells are reliant upon extracellular protein as their source of amino acids, mTORC activation prevents protein catabolism and thus inhibits cell growth. Furthermore, mTORC inhibition leads to enhanced protein catabolism, linking substrate availability to downstream protein synthesis.

While neutrophils in areas of inflammation may be subject to similar metabolic stresses as tumour cells, there are fundamental differences between tumour cells and neutrophils which must be considered. While a key fate decision for tumour cells is whether or not to proliferate, neutrophils are terminally differentiated, non-proliferating cells and so a more important outcome decision is likely to be survival versus apoptosis/necrosis. Both the rate of neutrophil cell death and the mechanism (i.e. apoptosis or necrosis) will have important effects on resolution of inflammation.

The pathways which regulate protein uptake and breakdown are potentially pertinent to neutrophil function in the inflamed lung, particularly given the

abundant protein leak described in the previous chapter. The role of hypoxia in regulating these processes is also of interest due to the close links between hypoxic signalling pathways and metabolic processes, including the mTOR pathway. mTORC1 dependent HIF activity is required to maintain glycolysis in effector T cells[181] and this axis also plays a role in trained immunity in myeloid cells[182]. In neutrophils, mTOR activity has been linked to formation of neutrophil extracellular traps (NETs) in a HIF-α dependent fashion[108]. mTOR has also been implicated in the regulation of neutrophil migration[183] and cytokine production[184]. Thus, mTOR signalling is clearly active in neutrophils but its role in regulating hypoxic responses and metabolism remains unclear.

The data described in chapter 3 demonstrates that active regulation of receptor expression and synthesis of inflammatory mediators are likely to underlie the hyperinflammatory phenotype seen in hypoxic neutrophils. The aim of this section is to determine the mechanisms which underlie this with particular focus on how these processes are fuelled.

Results

Inflammatory neutrophils are maximally glycolytic

I first sought to ascertain whether hypoxic neutrophils have an enhanced capacity for glycolysis compared to their normoxic counterparts. Despite a number of glycolytic enzymes being well described HIF target genes, I did not see any significant upregulation of glycolytic enzymes in the hypoxic neutrophil proteome (Fig 4.1A). I next investigated whether substrate availability was a factor in the glycolytic capacity of normoxic and hypoxic neutrophils. The glucose transporters Glut1 and Glut3 (SLC2A1 and SLC2A3) are also known to be HIF target genes. The proteomic data reveals that Glut3 is the dominant receptor in inflammatory neutrophils with expression levels very much higher than that of Glut1. Furthermore, Glut3 expression is significantly higher in hypoxic samples (Fig 4.1B). So, while hypoxia in the context of an inflammatory response does not elicit an increase in the abundance of glycolytic enzymes, it does increase glucose transporter protein expression, suggesting an attempt to improve substrate availability.

Figure 4.1

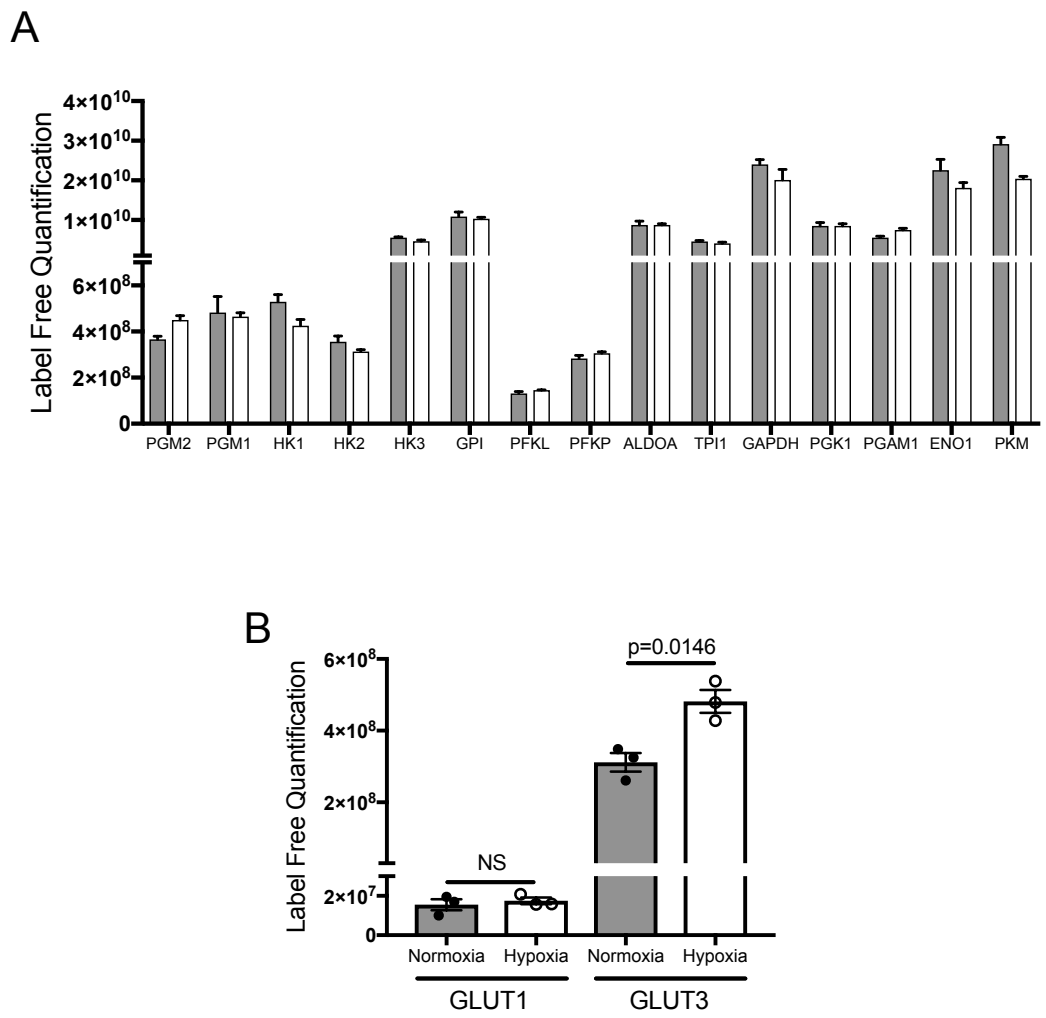


Figure 4.1: Hyperinflammatory hypoxic neutrophils have a distinct metabolic phenotype

(A) Label free quantification of (A) glycolytic enzymes and (B) glucose transporters in BAL neutrophils from normoxic (grey bars) and hypoxic (white bars) mice, 24 hours post-nebulised LPS. n=3, unpaired two tailed t-tests of significance. (A) Data represents mean ± SEM (B) Data represents individual values and mean ± SEM.

Glutamine, but not glucose is increased in hypoxic neutrophils

In order to investigate the metabolic status of normoxic and hypoxic neutrophils, I performed analysis of metabolite abundance by HPLC-MS in BAL neutrophils from normoxic and hypoxic mice 24 hours post-LPS. Despite the increase in glucose transporter expression described above, HPLC-MS did not demonstrate an increase in intracellular glucose in hypoxic neutrophils (Fig 4.2A). The airway is known to be a relatively glucose deplete environment[171]. I hypothesised that the unchanged intracellular glucose levels in hypoxic neutrophils, despite increased expression of glucose transporters, was due to low substrate availability. To investigate this, I measured glucose levels within the BAL fluid and found them to be extremely low with levels of less than 0.1mM (Fig 4.2B) compared to a blood glucose concentration in our model of approximately 12mM (data not shown). Our group has previously shown that regulation of glycogen stores occurs in response to knockout of the prolyl hydroxylase PHD2. Loss of PHD2 (and consequent enhanced HIFα accumulation, akin to hypoxic exposure) resulted in increased neutrophil glycogen stores. I therefore measured intracellular glycogen stores in freshly isolated BAL neutrophils (Fig 4.2C) and found no significant difference between normoxic and hypoxic BAL neutrophils.

I went on to investigate whether hypoxic neutrophils utilise an alternative energy source. In contrast to glucose and glycogen levels, intracellular levels of both glutamine and glutamate, measured by HPLC-MS are significantly higher in hypoxic neutrophils (Fig 4.2D). However, free glutamine was undetectable in BAL fluid (data not shown, lower limit of detection of assay

2nM). Given the data from tumour cell lines that scavenged proteins may provide a source of amino acids, including glutamine, and the increased extracellular albumin availability in hypoxic BAL fluid (Fig 3.2A) it was interesting to find significantly increased intracellular albumin levels in hypoxic neutrophils in the proteomic analysis (Fig 4.2E). This was an unexpected finding and was strongly suggestive of an active protein uptake pathway in these cells, not previously identified in neutrophils. This has proven to be essential in the hyperinflammatory phenotype observed in hypoxia and will be discussed in detail later in this chapter. I also examined the energy status of normoxic and hypoxic neutrophils using the HPLC-MS data. I found lower levels of ADP and ATP in hypoxic compared to normoxic neutrophils (Fig 4.2F). However, when the energy charge was calculated (a measure of the overall energetic status of cells) there was no difference between treatment groups (Fig 4.2G). These findings are challenging to interpret, while the lower ATP level may suggest depleted energy stores, the preserved energy charge (which is due to the lower ADP and AMP levels as well) may suggest that hypoxic neutrophils maintain lower levels of all three nucleotides, without compromising cellular energetics. Furthermore, these data represent only a snapshot of the energetic status at the 24-hour time point. Further experiments are required to understand the energetic status of neutrophils over the whole time-course of the inflammatory response.

In tumour cells, lack of available extracellular amino acids may be compensated for by uptake and breakdown of extracellular albumin. The high intracellular albumin and glutamine levels in hypoxic neutrophils suggested

that uptake (and breakdown) of proteins such as albumin from the extracellular environment may be a potential source of amino acids in neutrophils and I hypothesised that this amino acid source may provide substrate for central carbon metabolism.

Figure 4.2

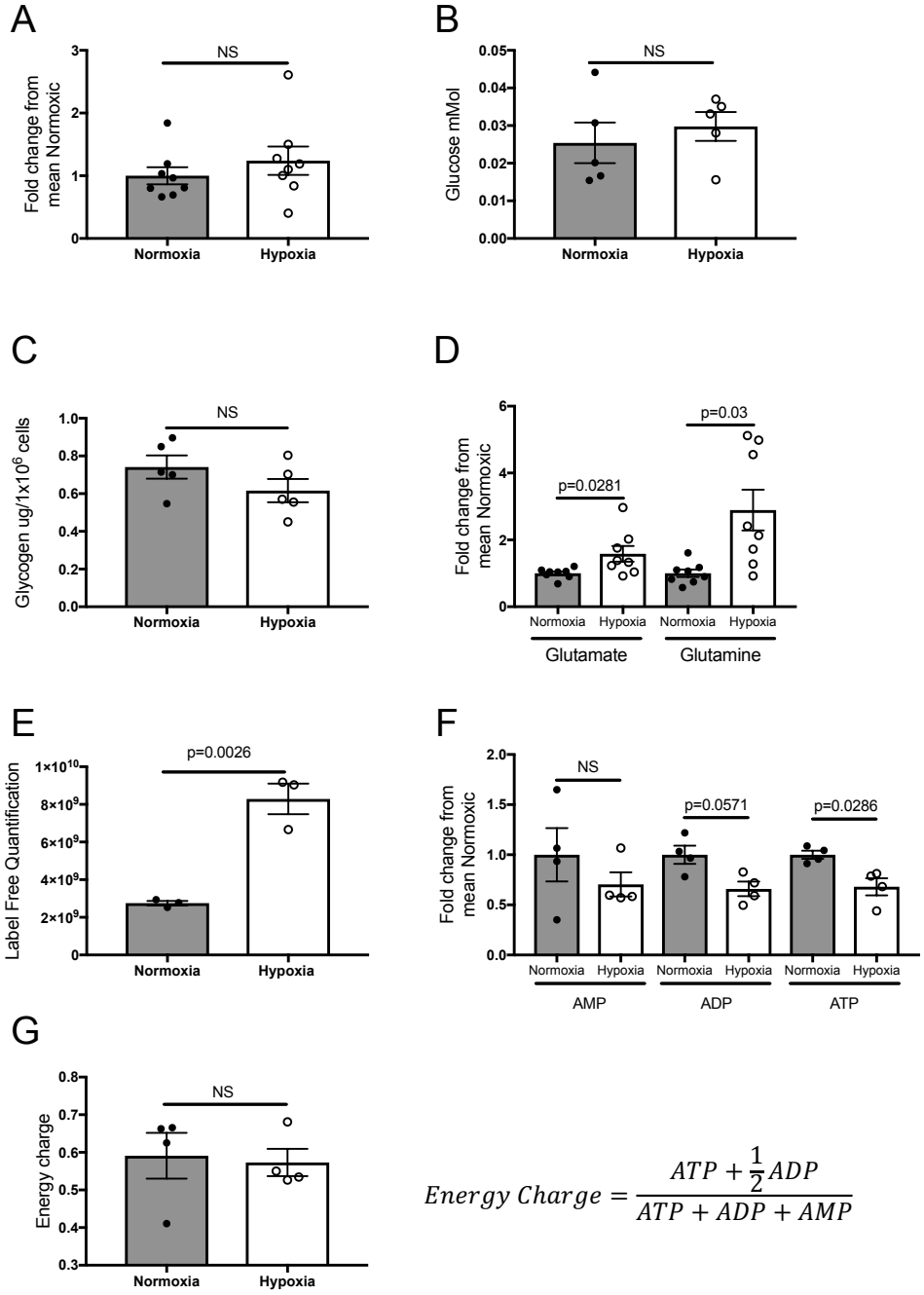


Figure 4.2: Hyperinflammatory hypoxic neutrophils have a distinct metabolic phenotype

Highly pure BAL neutrophils from normoxic and hypoxic mice, 24 hours post-nebulised LPS were analysed for (A) Intracellular glucose abundance, (D) glutamine and glutamate abundance, (F) AMP, ADP and ATP abundance and (G) energy charge by HPLC-MS and expressed as fold change from normoxic mean, n=8 over 2 experiments. The equation for calculation of energy charge is shown. (B) BAL supernatant glucose concentration measured by colourimetric assay, n=5, Mann-Whitney test of significance. (E) Label free quantification of albumin in BAL neutrophils from normoxic and hypoxic mice, 24 hours post-LPS, n=3. (A)-(D) and (F)-(G) Mann-Whitney test of significance. (E) unpaired two tailed t test of significance. The equation used to calculate energy charge is shown. Data represents individual values and mean ± SEM.

Hypoxia upregulates expression of proteins required for glutaminolysis

Supporting the hypothesis that in hypoxia, metabolism of glutamine is an important source of energy, a number of key enzymes and transporters involved in glutaminolysis are upregulated at the protein level in hypoxia (Fig 4.3A). These data also identified an important role for enzymes traditionally thought of as TCA cycle enzymes in the neutrophil. While the TCA cycle and downstream electron transfer chain are not used for ATP generation in the neutrophil, the proteomic data shows that they express the individual enzymatic components of this cycle (Fig 4.4A) but at lower levels than was seen for the glycolytic enzymes (Fig 3.4D). The HPLC-MS also shows that neutrophils express TCA cycle intermediates and, interestingly, show a trend towards higher levels of succinate which has been identified as a metabolite which also has immunomodulatory functions[89,160]. Lysosomal breakdown of proteins would be crucial to supply free amino acids from scavenged proteins. As noted in Fig 3.5C, the lysosomal pathway was enriched in those proteins which were up-regulated in hypoxia. I therefore hypothesised that increased lysosome activity may in part determine the metabolic and hyperinflammatory phenotype seen in hypoxia.

Figure 4.3

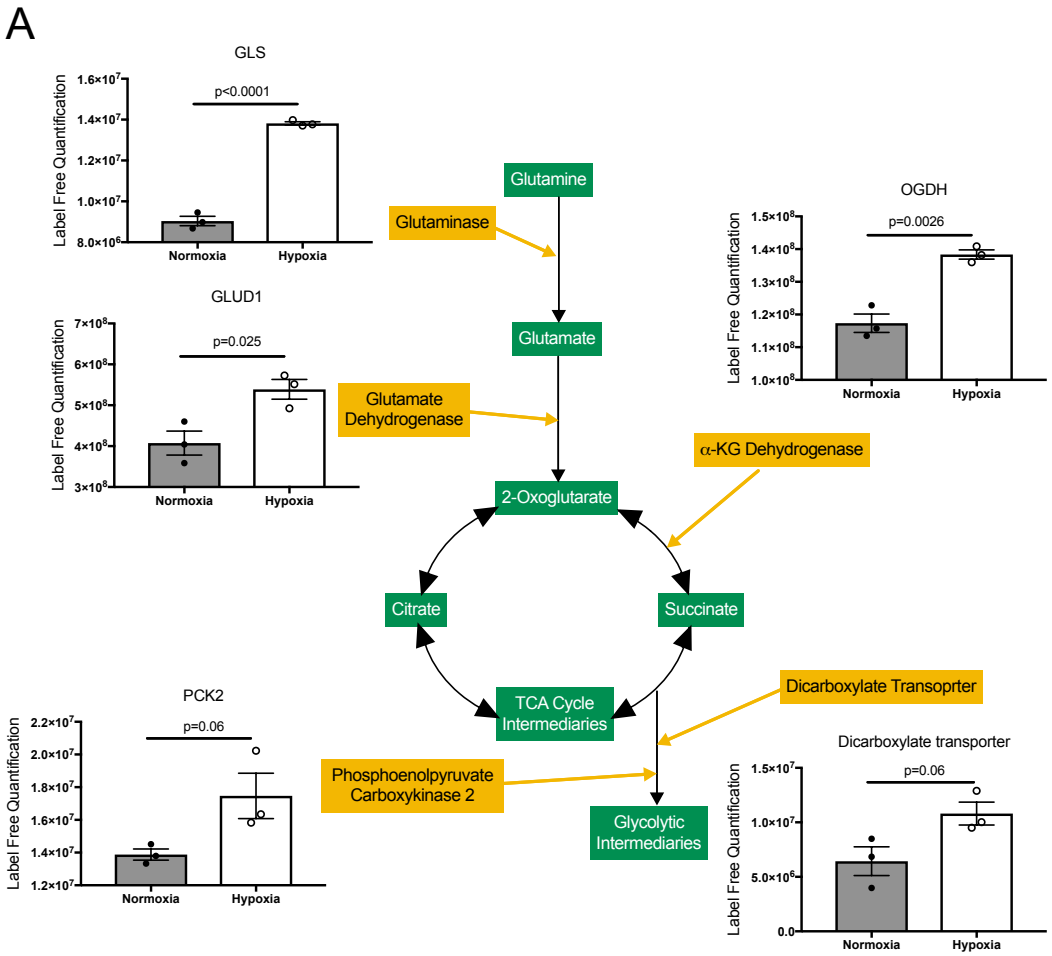


Figure 4.3: Hypoxic neutrophils show enhanced capacity for glutaminolysis
(A) Label free quantification of proteins involved in glutaminolysis in BAL neutrophils from normoxic and hypoxic mice, 24 hours post-LPS, n=3, unpaired two tailed t test of significance. Data represents mean ± SEM. Diagram represents the glutaminolysis pathway with enzymes in yellow and metabolic intermediaries in green. *GLS; Glutaminase, GLUD1; glutamate dehydrogenase 1, OGDH; 2-oxoglutarate dehydrogenase; PCK2; phosphoenolpyruvate carboxykinase*

Figure 4.4

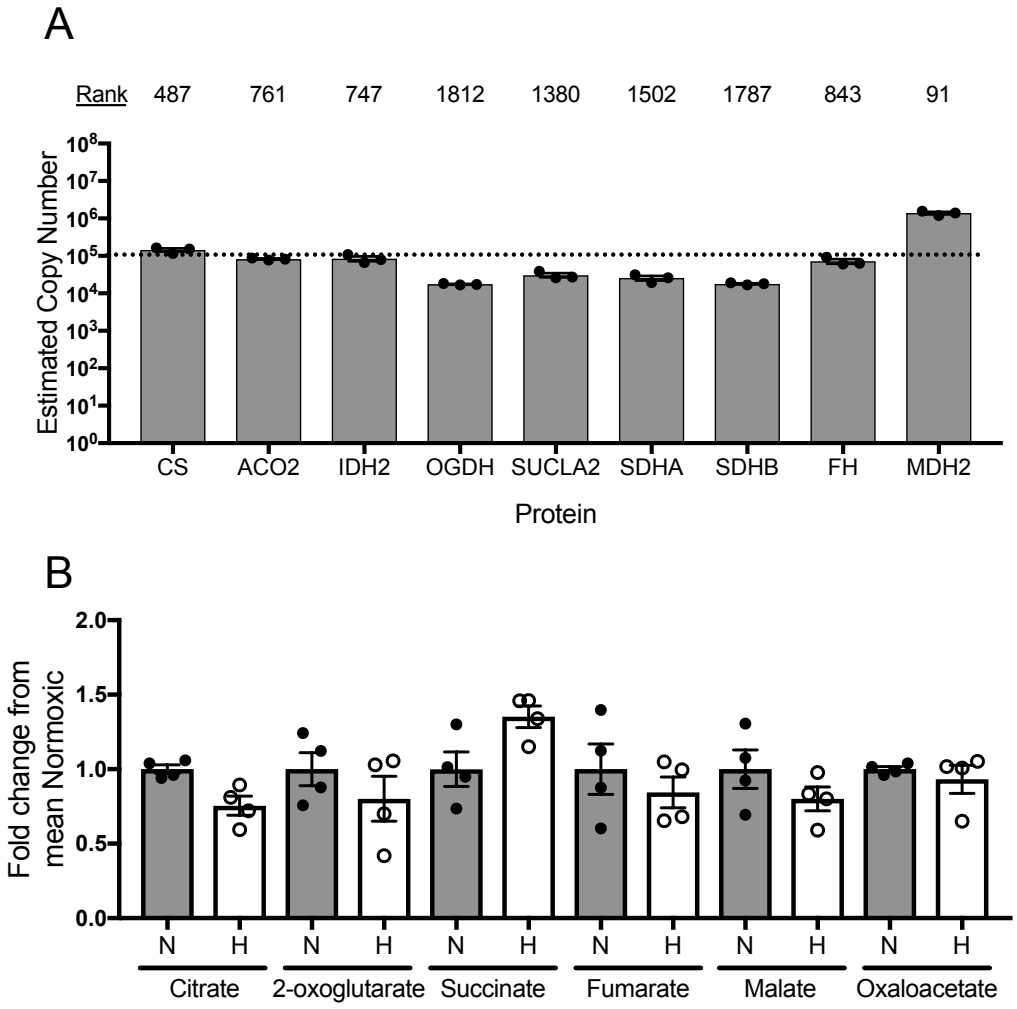


Figure 4.4: Neutrophils express TCA cycle enzymes and Intermediaries
(A) Estimated copy number of TCA cycle enzymes in BAL neutrophils from normoxic mice 24 hours post-nebulised LPS, n=3. The dotted line illustrates that most are present at fewer than 10^5 copies per cell. (B) Metabolite abundance measured by HPLC-MS in highly pure BAL neutrophils from normoxic and hypoxic mice 24 hours post-nebulised LPS, n=4, Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

Neutrophils scavenge and breakdown extracellular albumin

To investigate the uptake of extracellular proteins by neutrophils I utilised Texas Red fluorescently labelled bovine serum albumin (BSA) and double-quenched (DQ)-Green labelled BSA. The Texas Red albumin has constitutive fluorescence, but the DQ-Green BSA requires proteolytic cleavage of the quencher (usually in the lysosome) before it emits green fluorescence. Incubation of healthy human peripheral blood neutrophils with both Texas Red BSA and DQ Green BSA shows that uptake occurs quickly with a significant increase in Texas Red signal after just 30 minutes incubation, but the DQ-Green signal requires 90 minutes to significantly increase (Fig 4.5A). This supports the hypothesis that neutrophils can rapidly take up albumin from the extracellular environment and, over a longer time course, can break it down. As discussed previously, a key feature of the neutrophil anti-microbial response involves release of proteases into the extracellular environment. In order to confirm that the observed DQ-Green signal is a result of intracellular proteolysis (rather than extracellular proteolysis following degranulation, followed by uptake of unquenched, fluorescent BSA), the DQ-Green BSA time course was repeated but after 30 minutes of incubation with DQ-Green BSA the cells were washed to remove any residual extracellular albumin. Following a further 2-hour incubation in fresh media, there was a significant increase in intracellular fluorescence (Fig 4.5B), consistent with intracellular breakdown of DQ-Green BSA which had already been taken up.

In order to delineate the mechanism by which neutrophils take up albumin, I used confocal microscopy and measured co-localisation of fluorescently

labelled 70kDa dextran with FITC-BSA (Fig 4.5C). This large molecular weight dextran has previously been found to be taken up exclusively via macropinocytosis, i.e. fluid phase uptake[179]. The co-localisation of the FITC-BSA with the dextran is therefore suggestive of albumin uptake via macropinocytosis. This is consistent with the existing literature in other cell types.

Figure 4.5

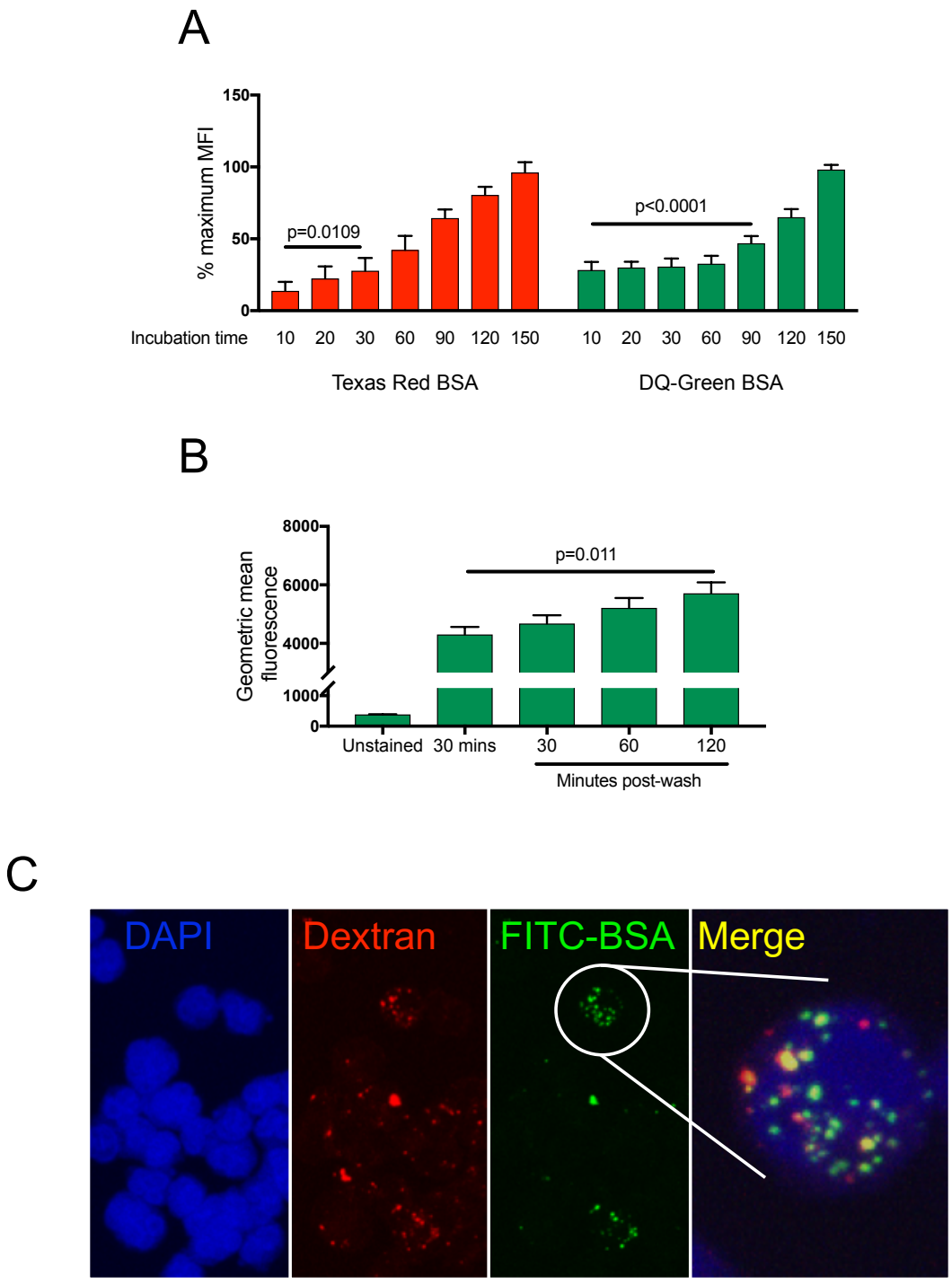


Figure 4.5: Neutrophils take up and breakdown extracellular proteins
(A) Time course of human neutrophils incubated with either Texas-red labelled BSA or DQ-Green BSA, data expressed as % maximum signal. (B) Geometric mean fluorescence of human neutrophils incubated with DQ-Green BSA for 30 minutes then washed and analysed for fluorescence over a further 120 minutes. (C) Confocal microscopy of neutrophils incubated with FITC-BSA (green) and 70kDa dextran (red) with DAPI nuclear staining in blue and red/green colocalization highlighted in yellow. (A)&(B) n=3, analysed for significance by one-way ANOVA with multiple comparisons. Data represents mean ± SEM.

Lysosomal proteins are upregulated by hypoxia

The lysosome would be predicted to be essential for breakdown of macropinocytosed proteins by neutrophils. In order to further interrogate the enrichment of the lysosomal pathway noted in figure 3.5C, I curated a list of known lysosomal proteins[185] and analysed their expression in the proteomes of normoxic and hypoxic neutrophils (Fig 4.6A and B). This highlights that the majority (although not all) of the known lysosomal proteins which were significantly different between groups were upregulated in hypoxia. These data suggest that in hypoxia (where substrate availability in the form of extracellular protein is high), neutrophils upregulate the whole pathway required for utilising these proteins, both the lysosomal components involved in proteolysis and, as shown in Fig 4.3, those enzymes required to metabolise the resultant free amino acids such as glutamine.

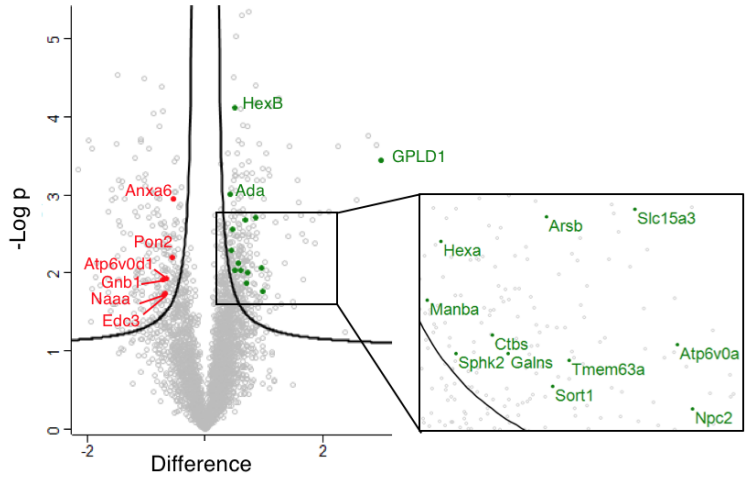
I went on to investigate the intracellular localisation of DQ-Green BSA using confocal microscopy. This demonstrated lysosomal trafficking of DQ-Green BSA as evidenced by staining for the lysosomal marker LAMP1 following incubation with DQ-Green BSA and identifying areas of colocalization (Fig 4.7A).

The central role of the lysosome is further established by the finding that manipulation of lysosomal activity alters the capacity of neutrophils to breakdown DQ-Green BSA: pre-treatment of healthy human peripheral blood neutrophils with the lysosomal protease inhibitor E64 (Fig 4.7B) or the inhibitor of lysosomal acidification chloroquine (Fig 4.7C) results in a small but significant reduction in DQ-Green signal

These data therefore confirm that neutrophils take up and breakdown extracellular proteins via the lysosome.

Figure 4.6

A



B

Protein names	Gene names	Fold change	P value
Phosphatidylinositol-glycan-specific phospholipase D	Gpld1	7.8093	0.0004
V-type proton ATPase 116 kDa subunit a isoform 2	Atp6v0a2	1.9209	0.0085
Epididymal secretory protein E1	Npc2	1.9188	0.0171
Solute carrier family 15 member 3	Slc15a3	1.8297	0.0019
CSC1-like protein 1	Tmem63a	1.6388	0.0100
Arylsulfatase B	Arsb	1.6018	0.0021
Sortilin	Sort1	1.5990	0.0133
N-acetylgalactosamine-6-sulfatase	Galns	1.5236	0.0093
Di-N-acetylchitobiase	Ctbs	1.4789	0.0076
Beta-hexosaminidase subunit beta	Hexb	1.4192	0.0001
Sphingosine kinase 2	Sphk2	1.4077	0.0093
Beta-hexosaminidase subunit alpha	Hexa	1.3850	0.0028
Beta-mannosidase	Manba	1.3549	0.0052
Adenosine deaminase	Ada	1.3432	0.0010
Annexin A6	Anxa6	0.6900	0.0011
Serum paraoxonase/arylesterase 2	Pon2	0.6839	0.0064
V-type proton ATPase subunit d 1	Atp6v0d1	0.6362	0.0119
Enhancer of mRNA-decapping protein 3	Edc3	0.6235	0.0185
N-acylethanolamine-hydrolyzing acid amidase	Naaa	0.6145	0.0190
Guanine nucleotide-binding protein subunit beta-1	Gnb1	0.6101	0.0120

Figure 4.6: Upregulation of the lysosome in hypoxic neutrophils
(A) Volcano plot of normoxic Vs hypoxic neutrophil proteomes with lysosomal proteins highlighted, significantly upregulated proteins are in green and down regulated in red, n=3, significance defined as $p<0.05$, $S_0=0.1$. Corrected for multiple hypothesis testing using permutation-based FDR=0.05. (B) Table showing details of significantly up- and down-regulated lysosomal proteins as detailed in (A) with p value and fold change shown.

Figure 4.7

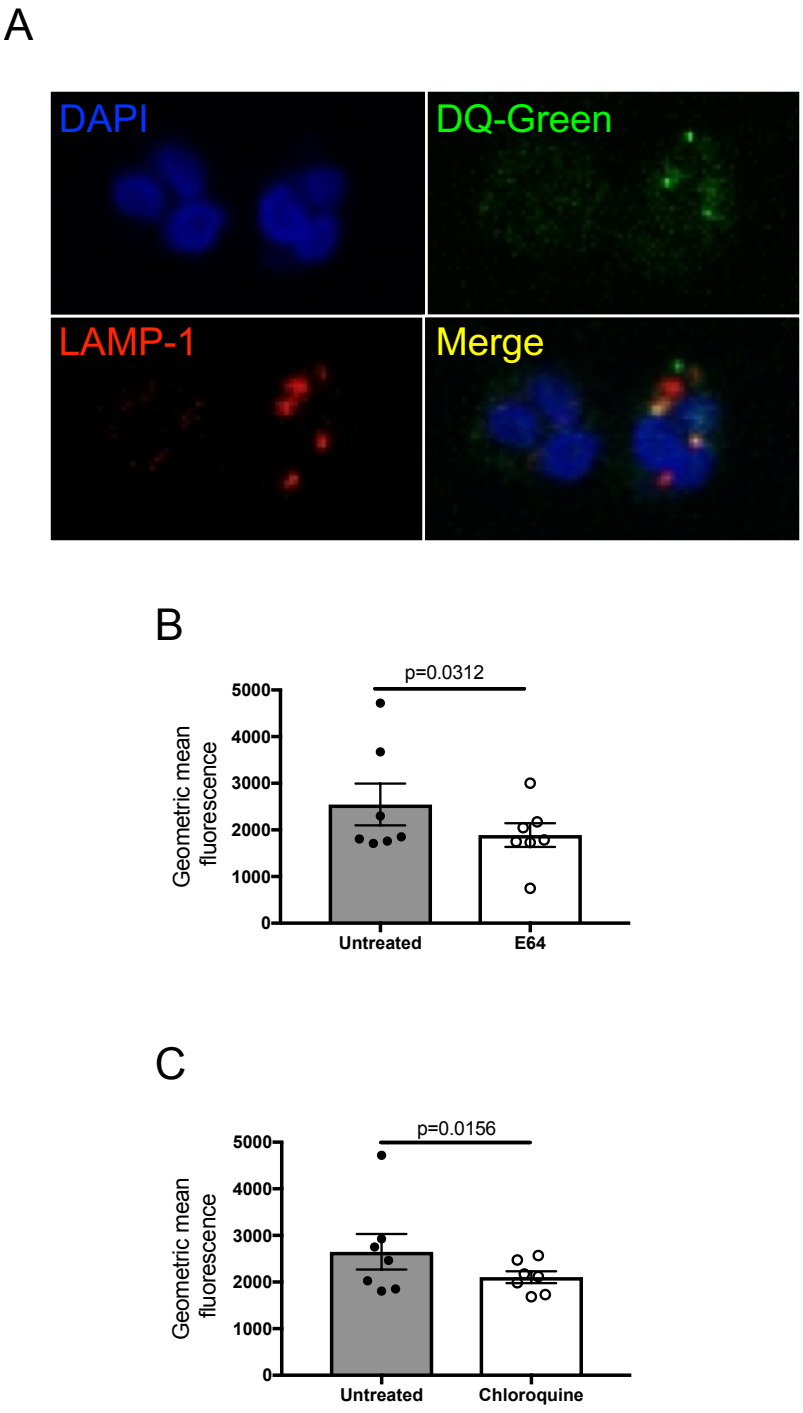


Figure 4.7: The lysosome is essential for neutrophil protein catabolism
(A) Confocal microscopy of neutrophils incubated with DQ-Green BSA (green)and stained for lysosomal marker LAMP1 (red) with DAPI nuclear staining in blue and red/green colocalization highlighted in yellow. Geometric mean fluorescence of LPS treated human neutrophils incubated with DQ-Green BSA and pre-treated with (B) the protease inhibitor E64 or (C) the inhibitor of lysosomal acidification chloroquine. n=7 over 3 experiments, Wilcoxon test of statistical significance for paired, non-parametric, data. Data represents individual values and mean ± SEM.

Uptake and breakdown of extracellular proteins is regulated by glucose and oxygen availability

I next sought to determine the factors which regulate the processes of protein uptake and breakdown in neutrophils. I hypothesised that nutrient availability, particularly glucose, may play a role in regulation of protein uptake and so incubated LPS treated human peripheral blood neutrophils in the presence or absence of glucose and compared normoxic (21% O_2) with hypoxic (1% O_2) culture. I utilised Texas-red labelled BSA to assess uptake and DQ-Green BSA to assess breakdown. The ratio of DQ-Green breakdown to Texas red uptake was used as an indicator of the breakdown activity of the cells. I found that hypoxic neutrophils in the presence of glucose, take up significantly more BSA than all other conditions (Fig 4.8A). In both normoxia and hypoxia, glucose free culture resulted in a significant reduction in Texas red albumin uptake. However, analysis of the DQ-Green signal in the same conditions showed that hypoxia significantly increased the signal, both in the presence and absence of glucose (Fig 4.8B). The ratio of breakdown to uptake showed that neutrophils incubated in hypoxia without glucose (conditions most closely resembling the hypoxic lung) had the highest breakdown activity (Fig 4.8C), consistent with the hypothesis that in conditions of nutrient scarcity, neutrophils exploit their environment to scavenge alternative energy supplies in the form of proteins and that hypoxia positively regulates this process.

Figure 4.8

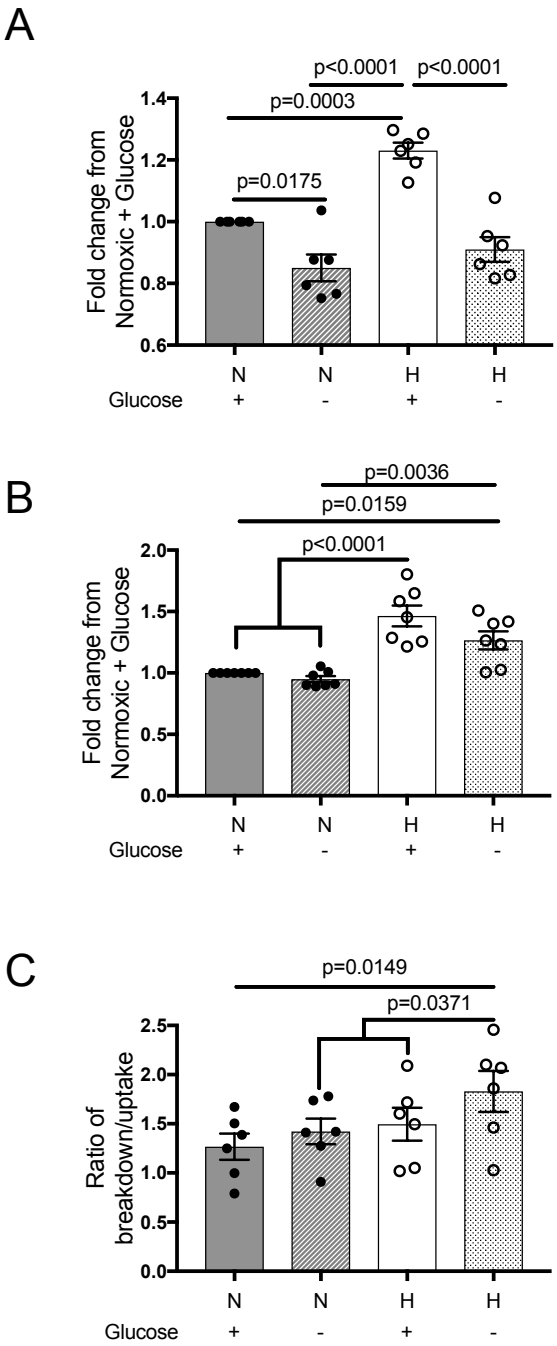


Figure 4.8: Oxygen and glucose availability regulate protein uptake and catabolism
Geometric mean fluorescence of LPS treated human neutrophils incubated with (A) Texas-Red labelled BSA (n=6) or (B) DQ-Green BSA (n=7) in normoxia or hypoxia (1% O_2) in RPMI ± glucose (11mM), data expressed as fold change from normoxia + glucose condition, data merged from 3 experiments. (C) Ratio of DQ-Green to Texas Red signal to illustrate breakdown efficiency in the conditions described in (A) and (B), n=6. Data represents individual values and mean ± SEM.

In vivo and *ex vivo* hypoxia regulate protein catabolism in inflammatory neutrophils

I then investigated whether the hypoxic upregulation of the lysosome pathway identified in our proteomic dataset corresponded to a change in protein uptake and breakdown by hypoxic inflammatory murine neutrophils. BAL neutrophils from normoxic and hypoxic mice were cultured *ex vivo* with fluorescent albumin compounds to measure uptake and breakdown. Neutrophils were isolated from the BAL 24 hours post-LPS induced lung injury and cultured *ex vivo* in glucose free media (to recapitulate the low glucose environment of the airway) in either hypoxia or normoxia. There were no significant differences in uptake of Texas red BSA between treatment groups (Fig 4.9A). Neutrophils from hypoxic mice, cultured in hypoxia *ex vivo* showed significantly higher DQ-Green signal (Fig 4.9B) than any other treatment group. Neutrophils from normoxic mice cultured *ex vivo* in hypoxia also showed a trend towards higher DQ-Green signal than when cultured in normoxia *ex vivo*, suggesting that while neutrophils from hypoxic mice have a pre-existing advantage in terms of capacity to take up and breakdown proteins, these pathways show a degree of plasticity and new hypoxic challenge *ex vivo* can further augment protein catabolism. Consistent with these findings, hypoxic neutrophils exposed to ongoing hypoxia show the highest breakdown to uptake ratio (Fig 4.9C). These data show that inflammatory neutrophils in glucose deplete, hypoxic conditions are most efficient at breaking down proteins which have been scavenged from the hypoxic environment.

Figure 4.9

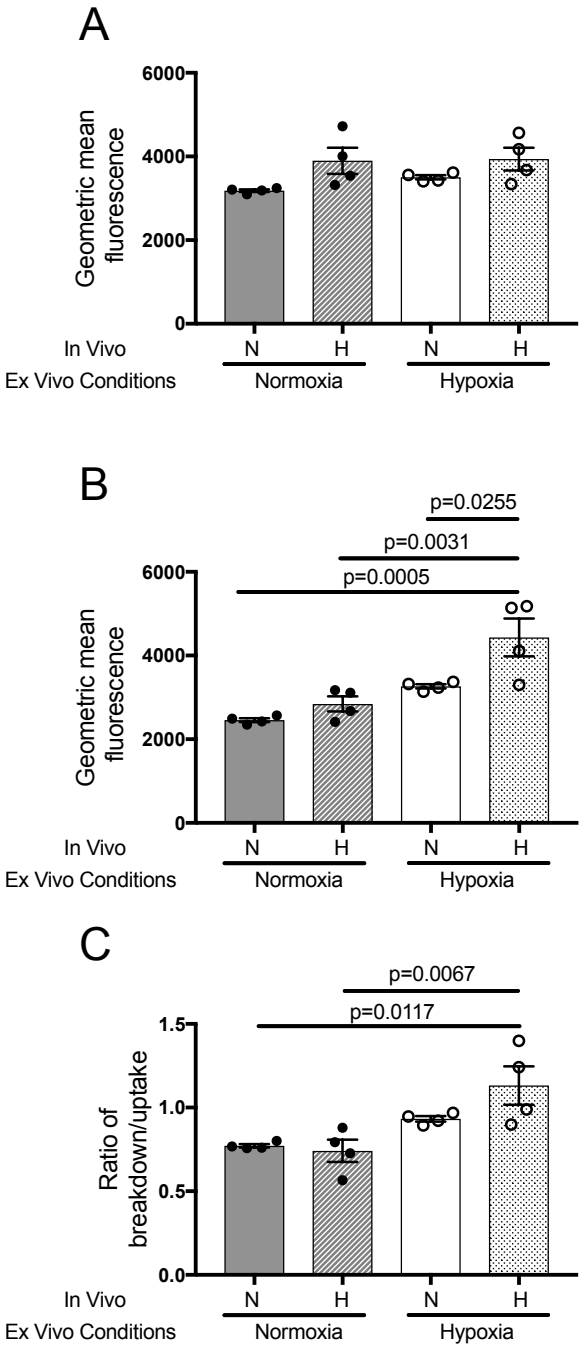


Figure 4.9: *In vivo* and *ex vivo* hypoxia regulate BAL neutrophil catabolic activity
Geometric mean fluorescence of murine BAL neutrophils isolated from normoxic and hypoxic mice 24 hours following LPS nebulisation and incubated with (A) Texas Red BSA or (B) DQ-Green BSA in glucose free media in normoxia or hypoxia (1% O_2). (C) Ratio of DQ-Green to Texas Red signal to illustrate breakdown efficiency in the conditions described in (A) and (B). (A)-(C) n=4, data represents individual values and mean ± SEM.

mTORC activity links oxygen and glucose availability to protein catabolism in neutrophils

The current literature suggests a critical role for the kinase complex mTORC in regulating lysosomal breakdown of proteins. I therefore investigated the role of mTORC activity in neutrophils. Our proteomic data confirms that neutrophils express the key components of mTORC1 but interestingly, the mTORC2 component RICTOR was suppressed completely in the hypoxic neutrophils(Fig 4.10A). Neutrophils also express the Ragulator complex proteins LAMTOR1-3 (Fig 4.10B) which are required for translocation of mTORC to the lysosomal membrane[186]. Neutrophil mTORC activity was measured by the phosphorylation status of the downstream target S6 kinase (S6K). The basal activity of mTOR in human peripheral blood neutrophils was analysed following culture with or without LPS stimulation. These data showed that, in unstimulated neutrophils, mTORC activity is low and LPS treatment results in a significant increase in the ratio of phosphorylated S6K ($p=0.0061$ by 2-way ANOVA, Fig 4.11A). Oxygen and glucose availability dramatically alter the activity of mTORC in human peripheral blood neutrophils treated with LPS (Fig 4.11B). Hypoxia and glucose deprivation independently significantly regulate mTORC activity in the LPS treated groups ($p=0.0015$ and $p<0.0001$ respectively by 2-way ANOVA). Glucose deprivation and hypoxia have an additive effect to almost completely suppress mTORC activity in hypoxic glucose free culture. When compared with the data presented in Fig 4.8, these data show that low mTORC activity correlates with efficient breakdown of extracellular proteins in our model. Furthermore, when mTORC activity was

assessed in BAL neutrophils from hypoxic and normoxic mice, mTORC activity was again suppressed in the hypoxic group (Fig 4.12A) although not to the same extent as was seen in the glucose deprived hypoxic human neutrophils. To investigate whether this was simply a correlation or whether mTORC activity was causative in regulating catabolism of extracellular proteins, human peripheral blood neutrophils were incubated with the mTORC activator MHY1485 or with the mTORC inhibitor Rapamycin prior to the addition of LPS and DQ-Green BSA. With mTORC activation there is a significant decrease in DQ-Green signal and with mTORC inhibition, there is a significant increase (Fig 4.12B). These data are consistent with the findings of Palm et al[174] that mTORC activity suppresses lysosomal breakdown of proteins.

Taken altogether, the data so far suggests that the hypoxic lung represents an optimal niche for excessive neutrophilic inflammation. Early degranulation results in significant early lung damage and as a consequence the extracellular environment is rich in protein. Hypoxic neutrophils show enhanced uptake of extracellular albumin and, in low glucose environments, enhanced lysosomal breakdown. This is likely to be due, at least in part, to suppression of mTORC activity and thus unfettered breakdown of protein in the lysosome. Additionally, there is upregulation of lysosomal proteins, further promoting breakdown of extracellular proteins.

Figure 4.10

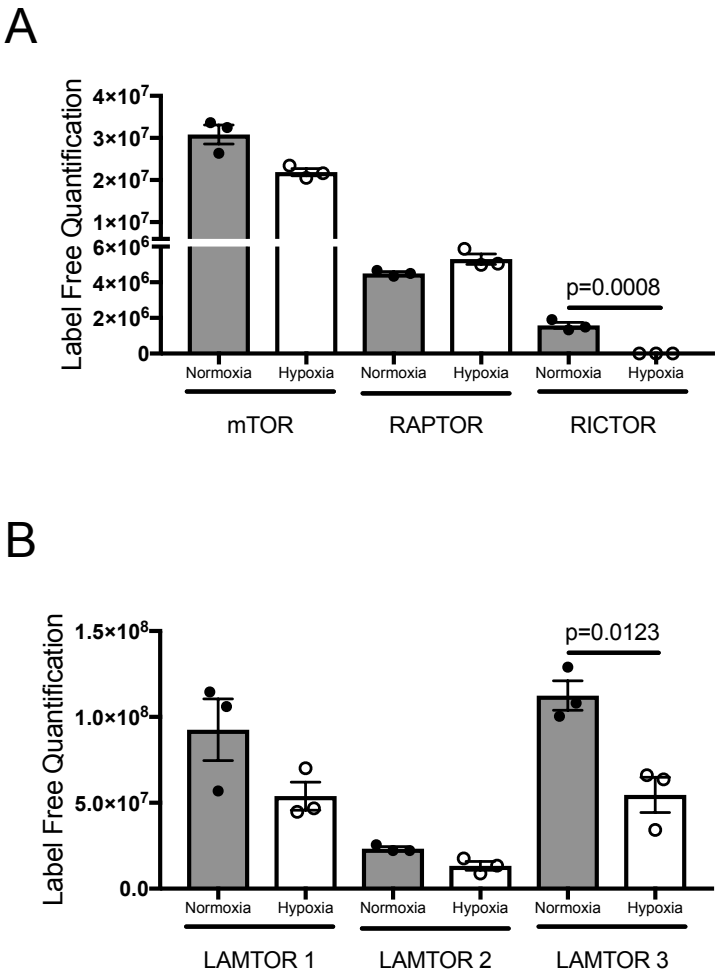


Figure 4.10: Neutrophils express mTORC machinery
Label free quantification of (A) mTORC components and (B) LAMTOR proteins involved in localising mTORC to the lysosome in highly pure BAL neutrophils from normoxic and hypoxic mice 24 hours post-nebulised LPS. (A)&(B) n=3, unpaired two tailed t test of significance Data represents mean ± SEM

Figure 4.11

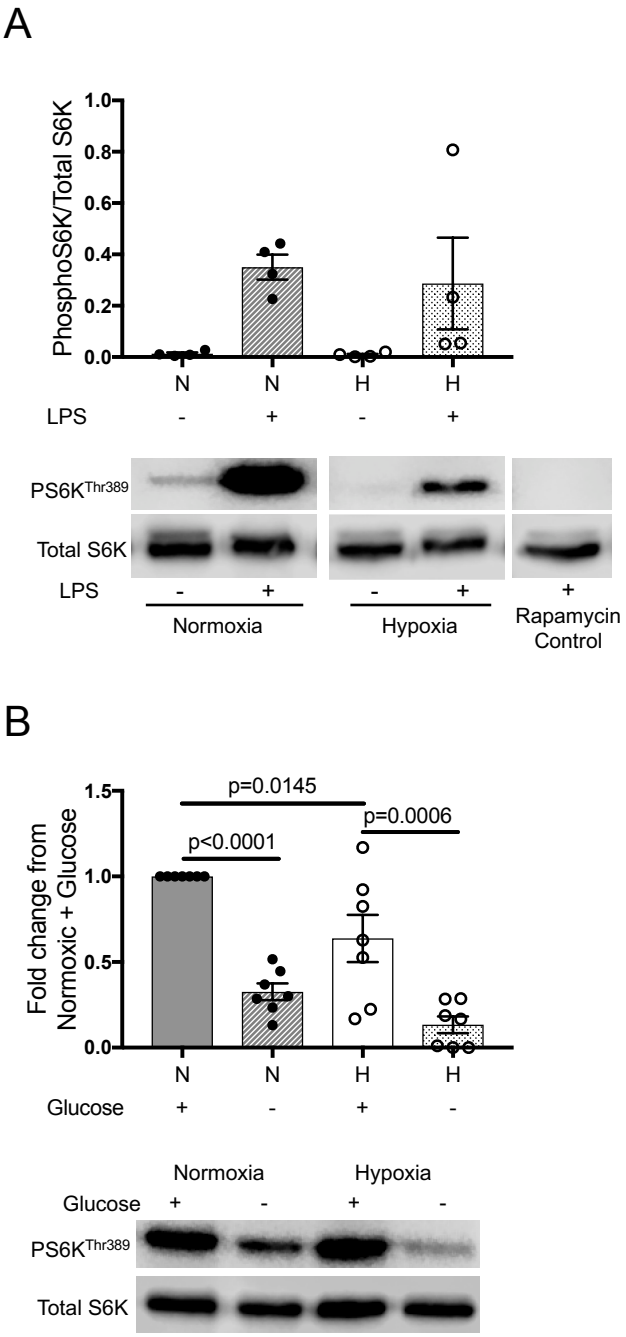


Figure 4.11: Neutrophil mTORC activity is regulated by LPS, glucose availability and hypoxia

Ratio of phosphorylated to total S6K measured by western blot in unstimulated and LPS treated human peripheral blood neutrophils cultured in normoxia or hypoxia (A). n=4 over 2 experiments, analysed by 2-way ANOVA with multiple comparisons, representative blot shown including rapamycin (mTOR inhibitor) control. (B) LPS treated human peripheral blood neutrophils were cultured in normoxia or hypoxia with or without glucose (11mM) and mTORC activity assessed by the ratio of phosphorylated to total S6K. n=7 over 4 experiments, data expressed as fold change from normoxia+glucose culture, representative blot shown. Analysed by 2-way ANOVA with multiple comparisons. Data represents individual values and mean ± SEM.

Figure 4.12

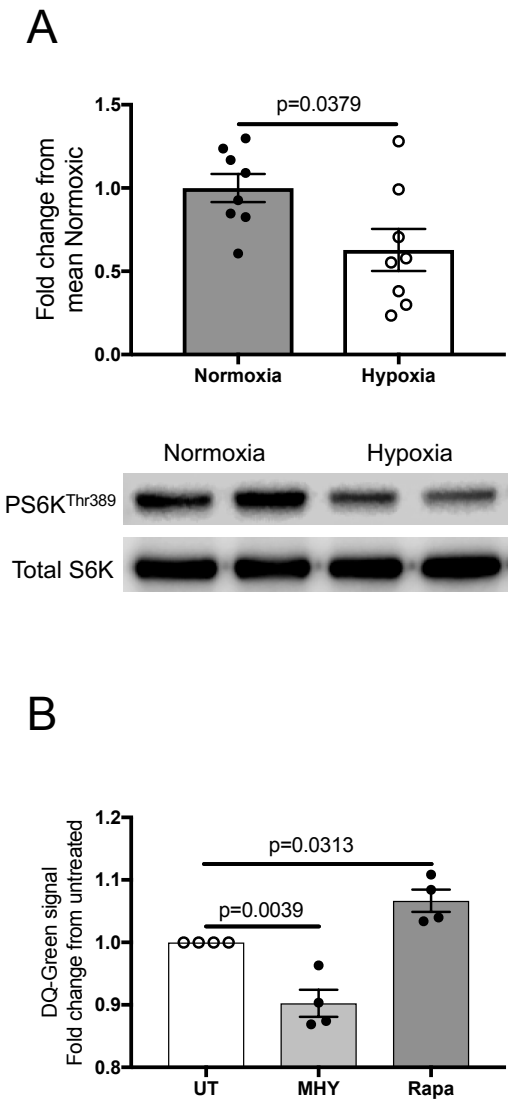


Figure 4.12: *In vivo* hypoxia results in reduced neutrophil mTOR activity
(A) Ratio of phosphorylated to total S6K measured by western blot in highly pure murine BAL neutrophils isolated from normoxic and hypoxic mice 24 hours following LPS nebulisation, n=8 over 2 experiments, Mann-Whitney test of significance, representative blot shown. (B) Geometric mean fluorescence of LPS treated human peripheral blood neutrophils cultured with DQ-Green BSA and MHY1485 (an mTORC activator) or rapamycin (an mTORC inhibitor), n=4 over 2 experiments, analysed by ordinary one-way ANOVA with multiple comparisons, data expressed as fold change from untreated control. Data represents individual values and mean ± SEM.

Glutamine from scavenged proteins can be metabolised by neutrophils

In order to test the hypothesis that extracellular proteins can be utilised by the neutrophil to fuel inflammation, I utilised labelled protein lysates from a cell line to investigate the fate of scavenged proteins. First, HEK cells which had been grown in media supplemented with $^{13}C_5$ labelled glutamine were lysed and the protein precipitated with acetone to remove any residual free labelled glutamine or metabolites. *Ex vivo* BAL neutrophils from hypoxic mice were cultured in media supplemented with the labelled protein lysates or with control (unlabelled) HEK cell lysates. Following 18 hours of culture in hypoxia, the neutrophils were harvested, lysed and HPLC-MS carried out to measure metabolite abundance and to identify the presence of metabolic intermediaries with heavy label incorporated. As illustrated in Fig 4.13A, label could be traced into TCA cycle metabolites, consistent with metabolism of glutamine into central carbon metabolism. With specific regard to 2-oxaloglutarate, this metabolite was only present in low levels in some samples, rendering the data unreliable. The graph shows those samples where there was sufficient metabolite to quantify label incorporation accurately (n=1 for control and n=2 for label) and is consistent with the data from other metabolites which were present at sufficient levels to include all samples.

It was also interesting to note that significant label incorporation occurred in UDP-GlcNAc (Figure 4.13B). This metabolite is the substrate for glycosyl transferases which regulate protein O-GlcNAcylation, an important post-translation modification with consequences for protein stability, activity and localisation[187]. Glutamine, as well as glucose, can be metabolised to UDP-

GlcNAc in T cells with important consequences for T cell functions[188]. Neutrophils have been found to have increased protein O-GlcNAcylation when stimulated with fMLF and the data presented here suggests that glutamine metabolism may be a source of UDP-GlcNAc in this process in neutrophils.

Figure 4.13

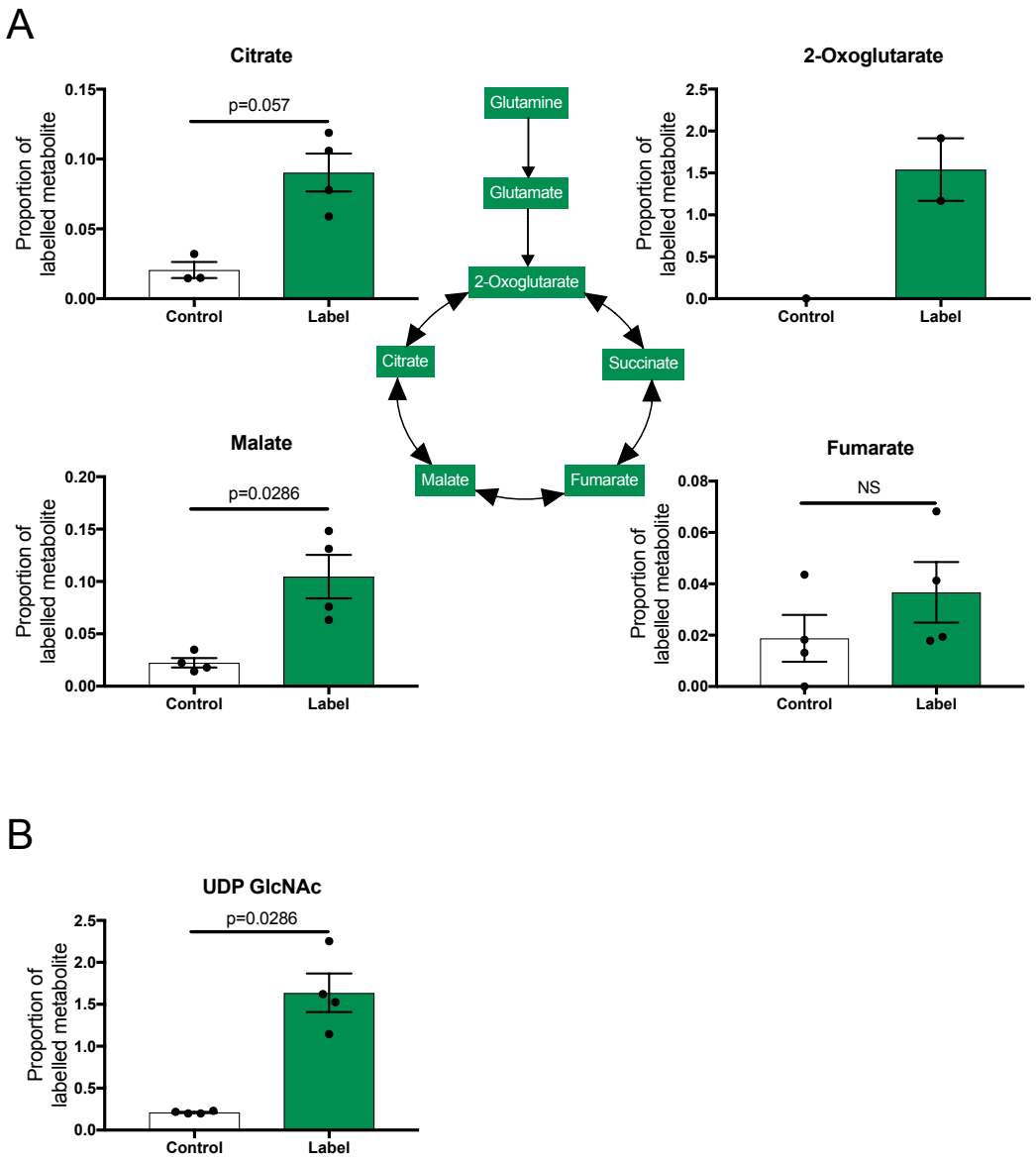


Figure 4.13: Glutamine from proteins can be traced into metabolic intermediaries
Highly pure BAL neutrophils harvested 6 hours post-nebulised LPS from hypoxic mice were cultured *ex vivo* for 18 hours in hypoxia (1%O_2) in glucose free media supplemented HEK cell lysates from unlabelled HEK cells (white) or HEK cells which had been cultured with 13C5 glutamine for 72 hours prior to lysis. Metabolite abundance and labelling was measured by HPLC-MS. n=4 (excluding 2-Oxaloglutarate, see text), Mann-Whitney test of significance. Data represents individual values and mean ± SEM.

In vivo chloroquine treatment attenuates neutrophilic lung inflammation

The data presented in this chapter demonstrate that neutrophils can take up and breakdown extracellular protein to use as fuel. *In vitro*, chloroquine inhibits this breakdown. Furthermore, chloroquine has been shown to improve outcomes in other acute lung injury models[189,190]. I therefore went on to carry out a pilot study to investigate whether chloroquine may have therapeutic value in an LPS induced lung injury and whether this was associated with a reduction in protein breakdown by lung neutrophils. In the published literature, chloroquine has been shown to affect recruitment of neutrophils to the lung, depending on the time frame of administration relative to the injurious stimulus. In order to isolate the effect of chloroquine on the behaviour of lung neutrophils, rather than on the recruitment of lung neutrophils, I trialled two different dosing schedules. Mice were injected IP with 50mg/kg of chloroquine diphosphate in 100μl of PBS or, for control mice, 100μl of sterile PBS alone. In the first dosing schedule this was done immediately following LPS nebulisation and in the second schedule the chloroquine was injected four hours post-LPS nebulisation. As in previous experiments, following nebulisation of LPS, mice were housed in either normoxia or hypoxia. Administration of IP chloroquine immediately following LPS nebulisation appeared to reduce neutrophil numbers in the BAL fluid 24 hours following LPS administration in both normoxic and hypoxic groups (Fig 4.14A). However, administration of chloroquine four hours after LPS stimulus does not alter neutrophil recruitment to the airways at 24 hours (Fig 4.14B). I therefore proceeded with this model. Analysis of peripheral blood cell numbers showed

no significant effect of chloroquine on circulating leucocyte populations when administered at the four-hour time point (Fig 4.14C). When BAL neutrophils were cultured *ex vivo* with DQ-Green albumin for two hours, there was a trend towards lower DQ-Green fluorescence in the chloroquine treated mice in both normoxia and hypoxia (although the effect was subtle in hypoxia) suggesting that IP chloroquine may be sufficient to reduce the lysosomal breakdown capacity of neutrophils recruited to the airways (Figure 4.14D). Whereas in previous experiments, BAL neutrophils from hypoxic mice had a higher DQ-Green signal than normoxic mice (Fig 4.9B), this was not the case in this experiment. It is therefore possible that the hypoxia/PBS treatment group have a sub-maximal DQ-green signal in this experiment for unknown reasons and the experiment will need to be repeated to resolve this discrepancy.

Importantly, analysis of the BAL supernatant also revealed a reduction in elastase activity in the chloroquine treated mice (Figure 4.14E). Again, the effect was subtle in the hypoxic mice and more marked in the normoxic mice. Finally, I measured the body temperatures of the mice immediately before they were culled (i.e. at the 24-hour time point). I found that, in normoxia, chloroquine treatment led to an increase in body temperature (Figure 4.14F). Due to a technical error, the temperature of the three out of the four hypoxia/PBS mice was not measured. I have therefore plotted this single measurement only however, it is consistent with the mean temperature of hypoxic mice at 24 hours in previous experiments (33.2°C, see Figure 3.1) and the temperature of the hypoxic, chloroquine treated mice was slightly higher than this (mean=34.6°C, Figure 4.14F). This preliminary data is consistent with

the literature which suggests that chloroquine can have a therapeutic benefit in models of acute lung injury. The less pronounced benefits in hypoxia may be due to the greater injury in these mice. Additionally, at the time of injection (four hours post-LPS) the hypoxic mice are more hypothermic than their normoxic counterparts and previous work from our group demonstrates that in an infection model, acute hypoxia results in lower blood pressure and lower cardiac output[143]. It is therefore possible that hypoxic mice do not achieve a therapeutic level of chloroquine as rapidly as the normoxic mice, resulting in a less marked effect of chloroquine treatment. While the reduced DQ-Green signal and supernatant elastase activity do not reach statistical significance here, this is likely to be due to the small number of mice in each group and increasing this number will be required to identify any significant differences between groups. Optimisation of the dose and schedule will be required to fully elucidate the effect and therapeutic potential of chloroquine in this model, but this preliminary data suggests this is worthy of further investigation.

Figure 4.14

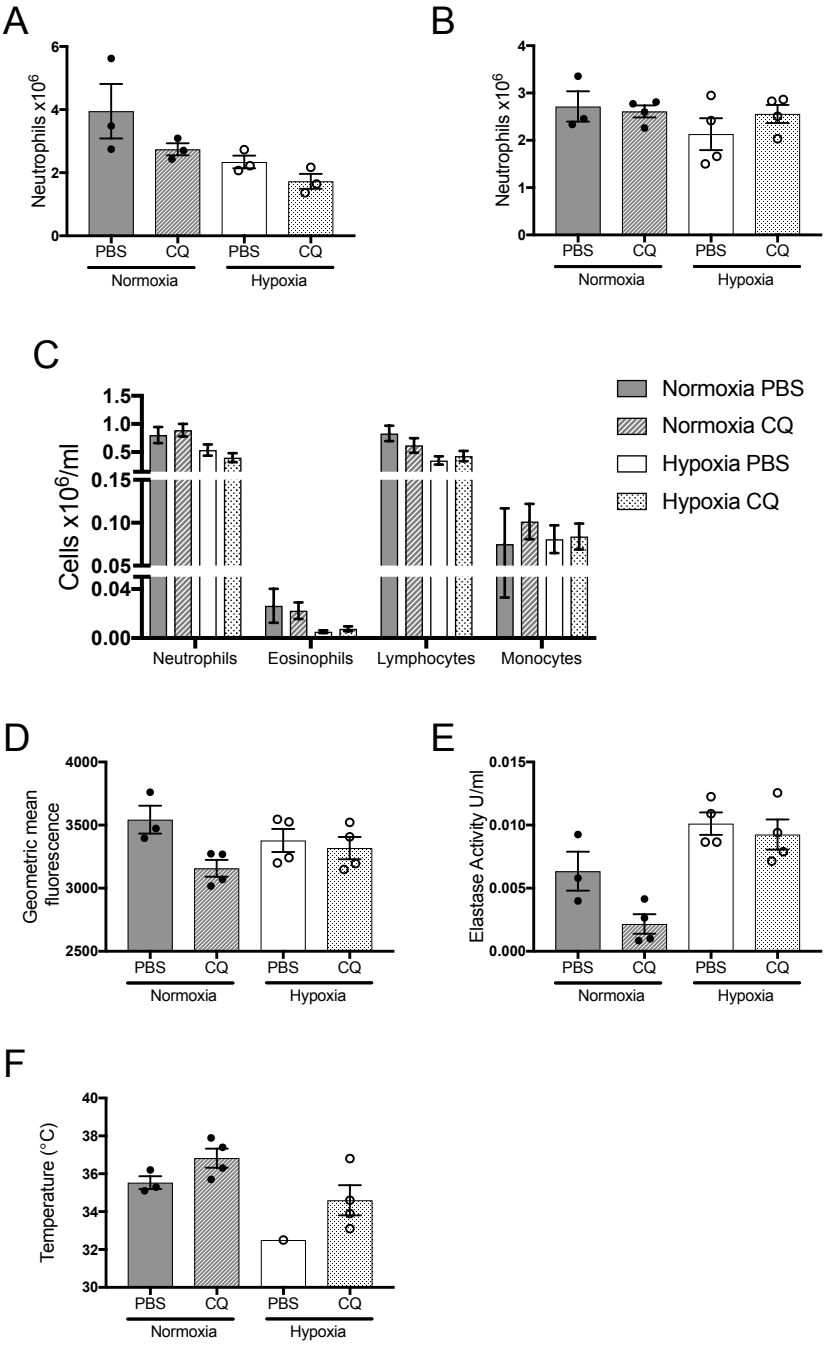


Figure 4.14: Chloroquine attenuates acute lung injury *in vivo*
BAL neutrophil counts from mice 24 hours following LPS nebulisation, treated with IP chloroquine or IP PBS at T_0 (A) or $T_{4\ hours}$ (B) and housed in normoxia or hypoxia, n=3-4. (C) Circulating blood leucocyte populations 24 hours following LPS nebulisation from mice treated with IP chloroquine or IP PBS at $T_{4\ hours}$ and housed in either normoxia or hypoxia. (D) BAL neutrophils incubated *ex vivo* with DQ-Green BSA and analysed by flow cytometry, (E) elastase activity in the BAL supernatant and (F) body temperatures at 24 hours. (D)-(F) in mice treated with IP chloroquine or IP PBS at $T_{4\ hours}$ and housed in normoxia or hypoxia, n=3-4. All data represents individual values and mean ± SEM except (C) where data represents mean ± SEM

Discussion

The lung represents a unique microenvironment: its role in gas transfer and its dual circulation result in highly variable oxygen availability, even in health. The lung is also immunologically interesting. It is constantly exposed to innocuous foreign material and failure of immune tolerance can result in significant pathology such as allergy, asthma or autoimmune disease. Conversely, the lung is susceptible to infections including bacterial, viral and fungal infections. This susceptibility to infections is often exacerbated by chronic inflammatory diseases such as cystic fibrosis, bronchiectasis and COPD which are all characterised by recurrent infectious exacerbations. There is increasingly evidence that the microenvironment of the lung is essential in maintaining effective immunity. In particular, the alveolar epithelium plays an important role in maintaining this microenvironment and inflammation may lead to perturbation. The low glucose concentrations within the airway are thought to be an important factor in maintaining the sterility of the airways and dysregulation of airway glucose levels, for example in diabetes mellitus may result in increased bacterial colonisation of the airways[191-193]. The data presented here suggest that neutrophils are highly adapted to function in this low glucose environment, despite their predisposition to generate energy via glycolysis. Furthermore, *in vitro* and *ex vivo* experiments suggest that the low glucose environment may itself alter neutrophil function.

Nonetheless, glycolysis is essential in fuelling neutrophils during the inflammatory response. The rate of glycolysis is dependent on a number of factors including abundance of glycolytic enzymes, enzymatic activity and

substrate availability. Hypoxia would be predicted to upregulate glycolysis because both glucose transporters and glycolytic enzymes are known HIF target genes, however, inflammatory stimuli can also stimulate glycolysis in neutrophils. The proteomic data set shows that, in the inflammatory environment, an additional hypoxic stimulus cannot upregulate glycolytic enzyme abundance in neutrophils. LPS has been shown to stabilise HIFα in both neutrophils[143] and macrophages[194] and so it is possible that neutrophils from normoxic mice undergo a degree of HIF stabilisation and thus induction of glycolytic genes which cannot be further induced by concomitant hypoxia. Hypoxia is, however, able to induce other HIF target proteins above the levels seen in normoxic inflammatory neutrophils including the glucose transporter Glut3. The quantitative nature of the proteomic data set identified Glut3 as the dominant transporter in BAL neutrophils. Both normoxic and hypoxic neutrophils did also express Glut1 but at very much lower levels. The Glut3 LFQ intensity translated to over 100,000 of copies of this protein per cell in hypoxia. Coupled with the very high expression levels of glycolytic proteins (as detailed in Figure 3.4D) in both treatment groups these data highlight the enormous glycolytic capacity of these cells. It is well established that neutrophils do not utilise oxidative phosphorylation for ATP generation[159]. It was therefore interesting to find that neutrophils express all of the essential enzymes for the TCA cycle, albeit at lower levels than glycolytic enzymes. The HPLC-MS metabolite data also showed the presence of TCA cycle intermediates in both normoxic and hypoxic neutrophils. These data raise the question of what the function of these proteins and metabolites is in

neutrophils. Studies of the neutrophils of patients with mutations in the SDHb gene demonstrated succinate accumulation and enhanced neutrophil survival as well as reduced oxidative stress, confirming a role for components of TCA cycle out-with energy generation in this cell type[160]. It is important to note that the TCA cycle is really a series of bidirectional enzymatic reactions whose directionality will be altered by multiple factors including enzyme activity and availability but also metabolite abundance. There is increasing interest in the role of metabolites in regulating immune cell function. Tannahill et al[89] identified succinate production by macrophages as a pro-inflammatory signal required for production of IL-1β. The metabolite S-2HG also has immunomodulatory functions in T cells[90] and HIF-1α regulates S-2HG accumulation[195]. Glutamine metabolism is required to generate both succinate and S-2HG. The data presented in this chapter demonstrate that glutamine is an important source of TCA intermediaries in inflammatory neutrophils and that the proteins involved in glutaminolysis are upregulated by *in vivo* hypoxia. Thus, while neutrophils may not utilise the TCA cycle for energy generation in the traditional sense, some of its components may be important in energy generation from glutamine and others may have important immunomodulatory roles. This is likely to be of particular relevance in the context of hypoxia. The prolyl hydroxylase enzymes PHD1-3 which hydroxylate HIFα in the presence of oxygen also require the TCA cycle intermediate 2-oxoglutatrate as a substrate. Furthermore, both succinate and fumarate can inhibit PHD function[196] and so hypoxic signalling is sensitive to levels of TCA cycle intermediaries (Figure 1.2). Hence, while the TCA cycle

may not be required for energy generation in neutrophils, its components are likely to play an important role in glutamine metabolism, immune function and the hypoxic response.

The parallels between the tumour microenvironment and the inflamed lung have proved useful for understanding some of the biological processes identified in hypoxic neutrophils. The identification of high intracellular levels of albumin in neutrophils was initially surprising but, in the context of the literature regarding albumin utilisation by tumour cells it led to the identification of important neutrophil behaviours which were modulated by hypoxia.

The finding that amino acids from scavenged proteins can be utilised for central carbon metabolism mirrors findings in tumour cells[173]. In tumour cell lines, Palm et al[174] found that mTOR was an important regulator of this process. Interestingly, the source of amino acids appeared to be important in directing mTOR signalling in this context: free amino acids resulted in mTOR activation and protein synthesis while amino acids from scavenged proteins inhibited mTOR activation . The data presented here show that, *in vitro*, glucose and oxygen availability regulate mTOR activity in neutrophils. Unstimulated blood neutrophils from healthy donors have very low levels of mTOR activity, as measured by the phosphorylation state of the S6 kinase. LPS stimulation induces mTOR activity in peripheral blood neutrophils but the degree of activation is clearly modulated by glucose availability and by hypoxia. In contrast, BAL neutrophils from LPS stimulated mice show basal mTOR activity which is moderately supressed in the BAL neutrophils of hypoxic mice (although not to the same degree as seen in glucose deprived

hypoxic human blood neutrophils). These data are consistent with the concept that, when nutrients are limited, switching off mTOR signalling allows unfettered catabolism of proteins in the lysosome. In the neutrophil population, low mTOR activity coincides with increased protein breakdown as measured by DQ-Green albumin fluorescence. Furthermore, experiments with chemical activation or inhibition of mTOR lead to reduced or increased fluorescence respectively which is consistent with a causative link between mTOR activity and protein catabolism in neutrophils. Another factor in the capacity of hypoxic neutrophils to catabolise scavenged proteins efficiently is likely to be their upregulation of lysosomal proteins. Hypoxia has been found to upregulate lysosomal proteins in colon carcinoma cells[197]. Additionally, hypoxia has been shown to be a positive regulator of the related process of autophagy in multiple cell types[198-200]. Investigation of *in vivo* hypoxia as an inflammatory stimulus in neutrophils has led to the identification of this process in neutrophils, however, while the data in this chapter are consistent with these processes being upregulated in hypoxic neutrophils, they are likely to occur to some extent in all inflammatory neutrophils. The hypoxic inflamed lung represents an ideal niche to promote protein uptake and catabolism.

Neutrophils have previously been considered a homogenous population of simple cells with limited capacity for protein synthesis. This paradigm meant that designing therapeutic interventions which could limit inflammation without obliterating immunity was extremely challenging and revolved around 'switching off' neutrophils. As we begin to understand the degree of heterogeneity in the neutrophil population, the capacity of neutrophils to adapt

to their environment and, critically, to continue to synthesise inflammatory mediators, this opens up a much wider range of therapeutic targets which could modulate neutrophil function in a more specific fashion. It also raises the interesting possibility of enhancing neutrophil function as well as limiting it. This may be useful in the context of tumour associated neutrophils or in chronic diseases such as COPD where persistent neutrophil infiltration is accompanied by recurrent infections, suggesting a degree of exhaustion in the neutrophil antimicrobial response. It is interesting to consider whether manipulation of the processes described here could be used to 're-arm' the neutrophil population to enhance immunity.

The data presented here suggest a number of novel therapeutic strategies which require further investigation. Firstly, the data suggests that manipulation of the lysosome may have potential therapeutic benefits in neutrophilic lung inflammation. The lysosome appears to be essential in generating the amino acid pool required for de novo synthesis of inflammatory mediators. There is some evidence that chloroquine may have therapeutic benefit in acute lung injury. Intraperitoneal injection of chloroquine prior to paraquat induced ALI resulted in reduced lung damage and lower numbers of infiltrating immune cells at 3 days as well as lower rates of lung fibrosis at 28 days[189], although the mechanism by which chloroquine was acting was not investigated. Pre-treatment with chloroquine was also protective in ALI associated with a rat model of pancreatitis[190]. Furthermore, both pre-treatment and delayed treatment with chloroquine proved protective in a mouse model of LPS induced sepsis. Chloroquine is most commonly used as an anti-malarial drug,

inhibiting parasitic haemoglobin digestion within erythrocytes[201]. However, it has also been utilised as an immunomodulatory agent in autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis and although the exact mechanism of action in this context is uncertain, it is postulated to be due to alterations in antigen processing. Chloroquine has also been found to reduce TNF-α production in macrophages[202]. Therefore this drug will have pleiotropic effects in acute lung injury which may include (but are highly unlikely to be limited to) suppression of neutrophilic protein catabolism. Systemic administration of chloroquine is likely to further muddy the waters. The data presented here suggest that IP chloroquine can attenuate LPS induced lung injury with evidence of a reduction in the capacity of lung neutrophils to catabolise protein in this context. It is possible that the benefit is not sufficient to overcome the major increase in lung injury caused by concurrent hypoxic exposure but also that absorption of the drug is limited in hypoxia due to the increased sickness of these mice and this requires further investigation. The timing of drug administration is also vital to consider. In patients, the onset and progression of ALI and ARDS are difficult to predict and so pre-treatment is rarely an option. A recent paper from Proudfoot et al investigating the potential of targeting TNF signalling in acute lung injury utilised a nebulised drug treatment with some benefit[203]. Thus, this form of 'topical' lung therapy may be useful in delivering a drug with multiple potential off-target effects to specifically target neutrophilic inflammation. Additionally, as chloroquine would be hypothesised to block the

persistent neutrophilic inflammation seen in hypoxia, rather than the onset of lung injury, delayed administration could still be therapeutic.

Secondly, these data highlight the potential of targeting metabolism to influence inflammation. The data raises interesting questions regarding regulation of neutrophil energetics by signalling pathways such as mTOR and AMPK, both of which have both been implicated in tumorigenesis. The inhibition of mTOR is consistent with increased catabolism of proteins but less consistent with ongoing protein synthesis. The time-course of the mTOR response in inflammatory lung neutrophils will be important to shed light on this apparent contradiction. It will also be important to investigate the intracellular localisation of mTOR as this is known to be important in sensing lysosomal signals.

In order to design therapeutic agents which specifically target neutrophilic lung inflammation, it is essential to understand how these cells behave in situ, and which factors regulate this. Examination of the neutrophil proteome has provided insights into the processes which are important in inflammatory neutrophils. The proteomic data confirmed that hypoxic exposure dramatically alters the neutrophil phenotype *in vivo*. Investigating the factors which contribute to hyperinflammatory phenotypes in hypoxia has identified novel neutrophil behaviours with important consequences for inflammation and resolution. The capacity of neutrophils to continue to synthesise inflammatory mediators following transmigration goes against existing dogma which states that granule proteins are synthesised and packaged in bone marrow neutrophils and can simply be released upon activation. These data also

highlight the degree of plasticity which exists and the adaptations which neutrophils are able to make in response to environmental stresses.

Chapter 5: PHD1 Regulates Neutrophil Metabolism and Survival with Consequences for Lung Inflammation

Introduction

Hypoxia profoundly alters the outcome of neutrophilic inflammation. The HIF transcription factors and their regulatory proteins the prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIH) are of vital importance in regulating cellular responses to hypoxia. As such, these proteins are of interest as therapeutic targets in neutrophilic inflammation. In a seminal paper, Cramer et al investigated the consequences of HIF-1α loss on myeloid cell function[75]. This study found that HIF-1α was essential for myeloid cells and that it regulated cellular energetics, bactericidal capacity, motility and migration. Additionally, HIF-1α is essential for neutrophil hypoxic survival[69], an important factor in the capacity of neutrophils to respond to infections in hostile inflammatory environments. HIF-1α is therefore not an optimal therapeutic target as its blockade is likely to result in failure of innate immunity. HIF-2α function has also been investigated in neutrophils and has been found to have a more specific role in neutrophil function. HIF-2α over-expression results in prolonged neutrophil survival as might be predicted and, consistent with this, loss of HIF-2α led to enhanced inflammation resolution due to increased neutrophil apoptosis at the site of inflammation[143]. This isoform selective function has also been identified within the PHD enzymes and, as such, it is hoped that manipulation of these proteins may allow more specific augmentation of hypoxic responses.

The isoform specific functions of PHDs in neutrophils are clearly illustrated by the very different phenotypes which are observed when PHD2 or PHD3 is knocked down. Loss of PHD2 led to enhanced neutrophil inflammation and failure of inflammation resolution, whereas loss of PHD3 resulted in loss of hypoxic survival in neutrophils, despite preserved HIF activity and this, in turn led to improved inflammation resolution.

The role of PHD1 in neutrophil function has not previously been investigated but PHD1 knockout has been found to be of benefit in a number of animal models, particularly in the setting of an ischaemic challenge. This therapeutic effect of PHD1 knockdown was first reported in a model of skeletal muscle ischaemia[204]. Using whole animal PHD1 knockout mice, the authors identified that PHD1 deficient skeletal muscle exhibited reduced oxygen consumption compared to WT mice. This reduction in oxygen consumption was due to an altered metabolic profile in PHD1 deficient myofibers with a switch from oxidative phosphorylation to glycolysis. This switch was mediated in part by upregulation of pyruvate dehydrogenase kinase (PDK) 1 and 4 which prevented entry of pyruvate into the TCA cycle. This adaptation allowed PHD1 deficient mice to tolerate an ischaemic injury to their hind-limb: increased glycolysis resulted in continued ATP generation and reduced oxidative stress in knockout muscle and therefore protection from the ischaemic necrosis which occurred in WT mice. PHD2 deficiency has also been shown to protect mice from an ischaemic limb injury but through a distinct mechanism, illustrating the isoform selective functions of these enzymes. $PHD2^{+/-}$ mice had a degree of “preconditioning” with increased collateral vessels at baseline (an adaptation

which was not present in PHD1 deficient mice) and a pre-adapted M2 macrophage population which triggered arteriogenesis[205]. Furthermore, genetic profiling of the macrophage population in PHD2 haplodeficient mice identified a shift to an M2 phenotype (M2 skewing) in $PHD2^{+/-}$ peritoneal macrophages with increased transcription of M2 markers including arginase1 and TGF-β.

PHD1 deficient mice were also found to be protected from a liver ischaemia/reperfusion injury, again due to reduced oxidative stress following reperfusion[206]. In keeping with the data from skeletal muscle, PHD1 deficient livers showed reduced oxygen consumption and increased PDK1 expression consistent with enhanced glycolysis. In both the ischaemic limb and liver injury models, $PHD1^{-/-}$ mediated protection was dependent on HIF-2α activity. More recently, $PHD1^{-/-}$ mice were shown to be protected from cerebral ischaemic injury. Neurons, like myofibers and hepatocytes, are ordinarily dependent on oxidative glucose metabolism. However, the protection conferred by PHD1 knockdown in this model does not rely on increased glycolysis, or on altered angiogenesis but instead on alteration in the redox status of hypoxic neurons and therefore enhanced ROS buffering capacity[207]. Interestingly, this phenotype is independent of HIF activity, unlike those described in the muscle and liver.

PHD1 has also been implicated in inflammatory processes. $PHD1^{-/-}$ mice were protected from dextran sulphate sodium (DSS)-induced colitis[208]. This protection was associated with improved intestinal epithelial barrier function due to enhanced survival of PHD1 deficient enterocytes. This in turn led to

reduced inflammation and reduced neutrophil infiltration. Furthermore, analysis of biopsies from patients with colitis found that PHD1 was upregulated in colitis and levels of PHD1 were correlated with inflammatory cytokine expression in patient biopsies[209]. In more recent studies from the Hindryckx group, haematopoietic cell PHD1 knockout was found to be required for protection against colitis[210]. The authors used a combination of Tie2Cre driven PHD1 deletion (which results in both endothelial and haematopoietic cell PHD1 loss) and bone marrow chimeras to identify that haematopoietic cell PHD1 deletion was necessary and sufficient to protect against colitis. In data which has interesting parallels with the studies of PHD2 haplodeficiency by Takeda et al[205], PHD1 deficiency resulted in skewing of the macrophage population to a more M2, anti-inflammatory phenotype. Taken together these data illustrate that specific functions may be attributed to each PHD isoform and that there is a lack of redundancy within the system. However, it is important to note that in some specific scenarios, loss of one isoform may be compensated for by another. In the paper by Takeda et al, when using a myeloid specific PHD2 deletion model, haplodeficient macrophages exerted the protective effect but PHD2 null macrophages did not. This was found to be due to a significant, compensatory uplift in PHD3 transcript in the PHD2 null macrophages. Additionally, in a study of PHD mediated regulation of T cell development, Clever and colleagues[211] found that knock down of all three PHD isoforms in $CD4^+$ T cells regulated lung inflammation. Specifically, PHD proteins were required to restrain proinflammatory Th1 cell function and to promote regulatory T cell (T_{reg}) development in response to innocuous immune

stimuli. This effect was not mediated by a specific PHD isoform, rather deletion of all three was required, consistent with context specific redundancy in the system.

The evidence above that PHD1 may regulate inflammatory responses in addition to hypoxic responses led us to consider whether PHD1 may play a role in neutrophilic inflammation. Given the previous work from our group demonstrating a specific role for PHD2 and PHD3 in regulation of neutrophil function which was distinct from their role in modulating macrophage behaviour, we elected to use a neutrophil specific PHD1 knockout model. In order to investigate the specific role of PHD1 in the neutrophil population, I have generated a mouse line with neutrophil-specific PHD1 knockdown. This utilised Cre-Lox technology as described in the methods section. Mice with LoxP sites flanking the PHD1 gene ($PHD1^{fl/fl}$) have been previously described[210] and these mice were crossed with a mouse line expressing the Cre recombinase under the transcriptional control of MRP8 (also known as S100-A8) with IRES mediated co-expression of green fluorescent protein (GFP). This line has been shown in the literature to selectively knock down the floxed protein in neutrophils[76,111,212]. This specificity is driven by the very high levels of expression of S100-A8 in neutrophils, however, it is important to acknowledge that although this protein is predominantly expressed in neutrophils, it is also expressed (albeit at lower levels) in myeloid and epithelial cells and so, like most Cre-Lox driven deletions, cannot be said to be completely specific. All mice were on a C57/Bl6 background.

The aims of this chapter were to characterise neutrophil specific PHD1 deficient mice and to investigate the consequences of neutrophil specific PHD1 loss for inflammatory responses in both normoxia and hypoxia. I hypothesised that loss of PHD1 would result in metabolic adaptations in the neutrophil population and that this would have knock-on consequences for neutrophil survival and therefore inflammation resolution.

Results

MRP8 driven Cre recombinase allows neutrophil specific deletion of PHD1

I first investigated whether MRP8 driven Cre expression in $PHD1^{fl/fl}$ mice did result in neutrophil specific loss of PHD1. Highly pure bone marrow neutrophils were isolated from $PHD1^{f/f}$ $MRP8Cre^{-/-}$ (wild-type, WT) and $PHD1^{f/f}$ $MRP8Cre^{+/-}$ (knockout, KO) mice. I carried out TaqMan analysis of gene expression using commercial primer/probe sets. This confirmed knockout of PHD1 mRNA in $Cre^{+/-}$ mice with no alteration in expression levels of PHD2 or PHD3 (Fig 5.1A). Bone marrow mononuclear cells were isolated and cultured until matured into BMDMs, as assessed by surface expression of F4/80 (Fig 5.1B). These BMDMs were then analysed for gene expression of PHD1, 2 and 3. This confirmed no reduction in PHD1 expression in the BMDMs from KO mice and no changes in expression of PHD2 or 3 (Fig 5.1C).

In order to assess knockdown of PHD1 in blood and lung neutrophils, I took advantage of the co-expression of GFP in the MRP8Cre positive cells. Using flow cytometry to measure GFP positivity, I found that 93% of peripheral blood neutrophils were GFP positive in KO mice while <1% of WT neutrophils were GFP positive (Fig 5.2A). This pattern held true for BAL neutrophils with 92.5% of BAL neutrophils being GFP positive in KO mice compared to <3% of WT BAL neutrophils (Fig 5.2B). Additionally, only 6% of peripheral blood monocytes were GFP positive in KO animals, and <2% of BAL macrophages were GFP positive in KO animals indicating neutrophil specific Cre expression in both the peripheral blood and BAL.

Figure 5.1

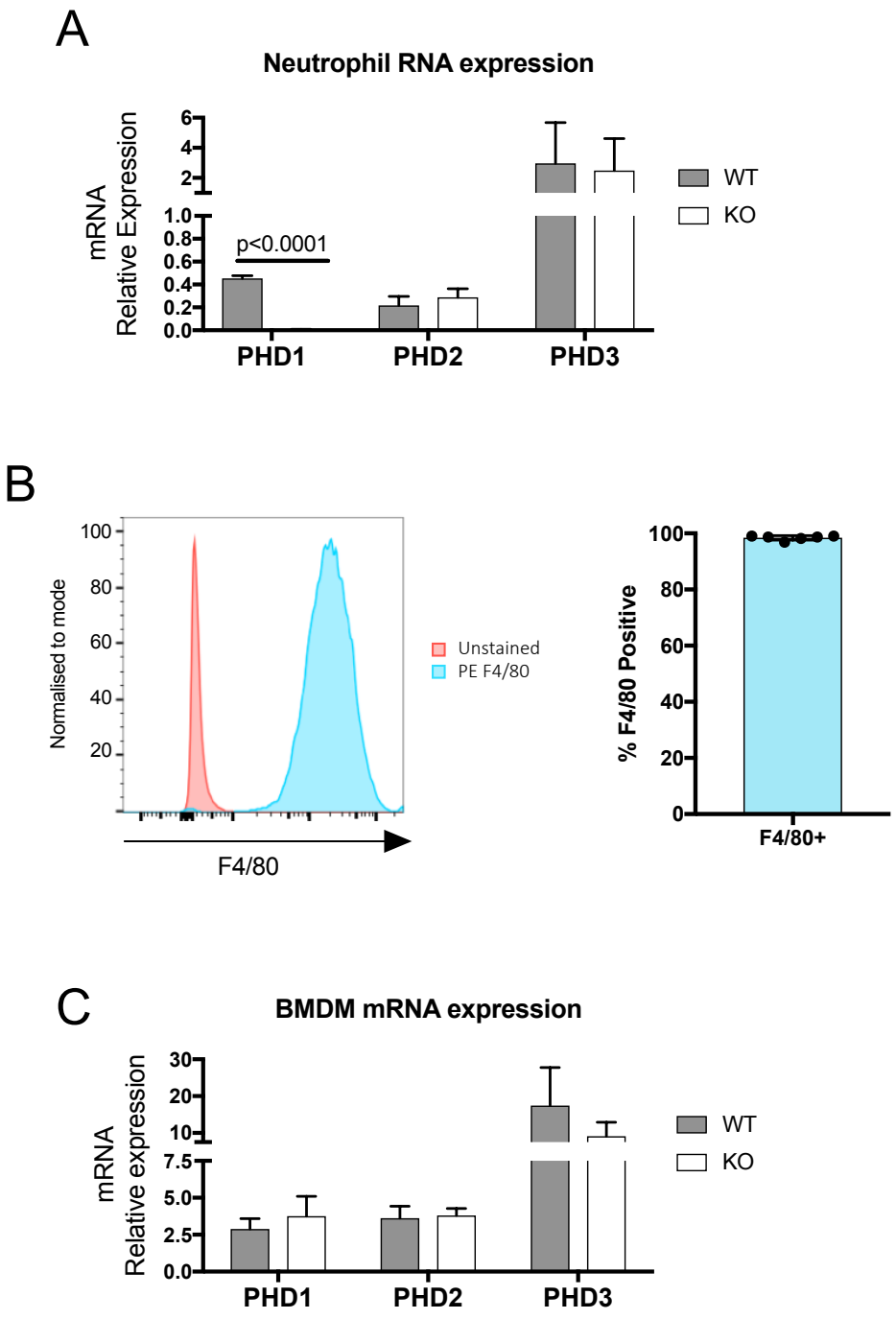


Figure 5.1: PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice display neutrophil specific loss of PHD1 mRNA in the bone marrow
TaqMan analysis of gene expression for PHD1 (*egln2*), PHD2 (*egln1*) and PHD3 (*egln3*) in (A) highly pure murine bone marrow neutrophils n=3-4. (B) Bone marrow derived monocytes (BMDMs were differentiated from bone marrow mononuclear cells and analysed by flow cytometry for expression of the monocyte marker F4/80, n=6. Bone marrow derived monocytes from wild-type (PHD1$^{fl/fl}$MRP8Cre$^{-/-}$) and KO (PHD1$^{fl/fl}$MRP8Cre$^{+/-}$) mice were subsequently analysed for gene expression for PHD1 (*egln2*), PHD2 (*egln1*) and PHD3 (*egln3*), n=3, analysed by unpaired t test. Data represents mean ± SEM.

Figure 5.2

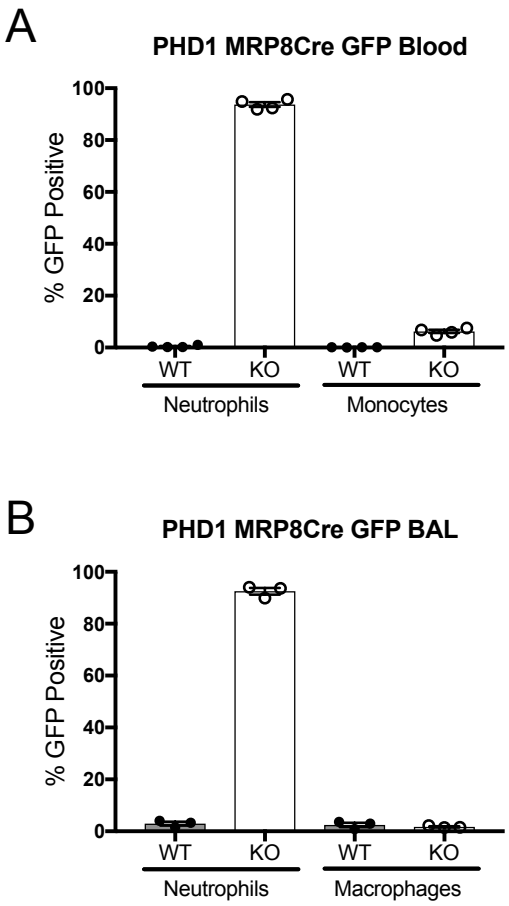


Figure 5.2: PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice display neutrophil specific knock out of PHD1 in blood and BAL neutrophils
Flow cytometry analysis of GFP positive and negative myeloid cells in the blood (A) and BAL (B) of WT and KO mice. Blood leucocytes were identified by CD45 positivity and blood monocytes by CD115 positivity. In both blood and BAL neutrophils were identified by Ly6G positivity. N=3-4, data represent individual data points and the mean ± SEM.

Neutrophil specific PHD1 deficient mice have normal circulating leucocyte populations

I went on to characterise the impact of PHD1 deletion on the behaviour and function of neutrophils. In order to establish that they had normal immune cell maturation and recruitment, I analysed leucocyte numbers and differentials in mice with or without an inflammatory stimulus. Whole blood was collected from WT and KO mice and analysed by flow cytometry for leucocyte differential counts. There was no difference between genotypes in the percentages or absolute numbers of circulating leucocytes in naïve mice (Fig 5.3A&B) or in mice 24 hours post-nebulised LPS challenge (Fig 5.3C&D). In both genotypes, LPS challenge results in a significant increase in the number of circulating neutrophils (Fig 5.3E).

Figure 5.3

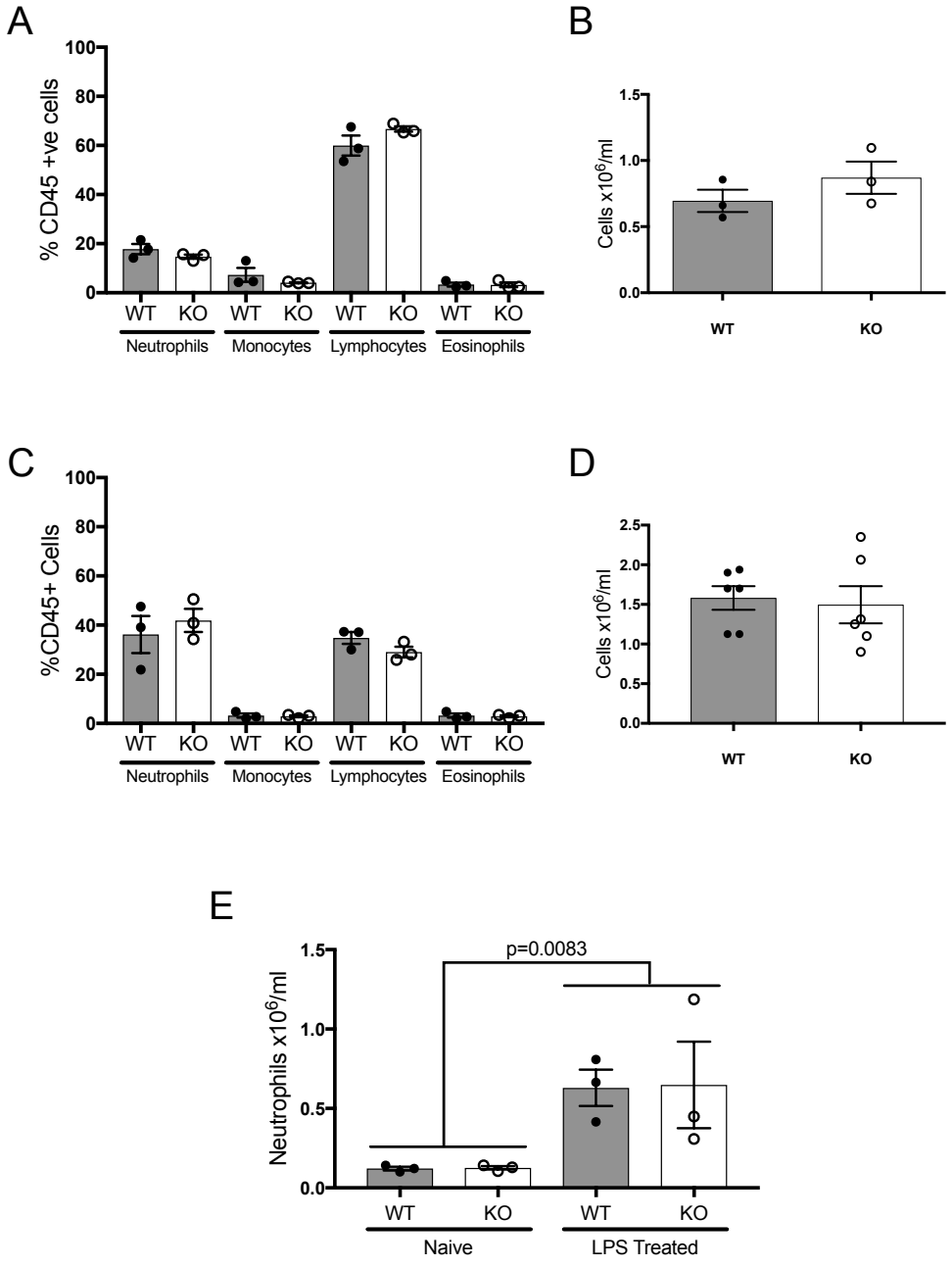


Figure 5.3: PHD1 deficiency does not alter circulating leucocyte populations
Whole blood from WT and KO mice was analysed by flow cytometry to establish the make-up of the leucocyte population and counted using a haemocytometer to establish absolute leucocyte counts. The % of each leucocyte population and absolute leucocyte count are shown for naïve mice (A)&(B) and for mice 24 hours after nebulised LPS (B)&(C). The absolute neutrophils numbers were calculated for WT and KO mice in both unstimulated and LPS treated animals (E), analysed by 2-way ANOVA. (A)-(C) and (E) n=3, (D) n=6 over 2 experiments. Data represent individual data points and the mean ± SEM.

PHD1 deficient neutrophils are functionally competent

Having established that neutrophil PHD1 deficiency does not alter circulating leucocyte numbers, I went on to investigate what, if any, the functional consequences are of PHD1 loss for the neutrophil. Inflammatory BAL neutrophils were isolated from WT and KO mice, 24 hours following nebulised LPS. Chemotaxis towards the mouse chemokine KC (CXCL1) was measured using ChemoTx plates (Neuro Probe). As Figure 5.4A shows, there was a dose dependent increase in chemotaxis in response to KC but no difference between the genotypes.

Phagocytosis was measured using fluorescently labelled heat-killed E Coli. This experiment was carried out using whole animal $PHD1^{-/-}$ mice (KO_{PHD1}) with C57/Bl6 wild-type controls rather than the $PHD1^{fl/fl}$ MRP8Cre mouse line. There was no difference between genotypes in their capacity to phagocytose heat killed bacteria (Fig 5.4B).

Thus, loss of PHD1 does not specifically alter neutrophil homeostasis or recruitment from the bone marrow following stimulation and PHD1 deficient neutrophils are functionally competent

Figure 5.4

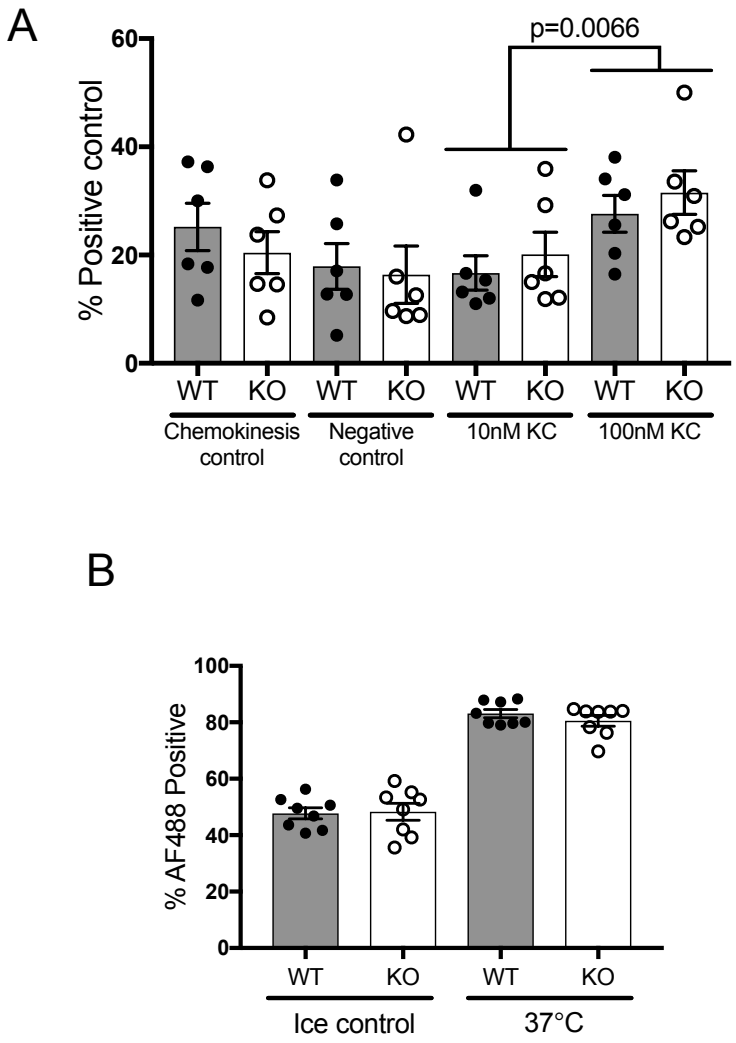


Figure 5.4: PHD1 deficient neutrophils are functionally competent
BAL neutrophils were isolated 24 hours after nebulised LPS from WT and KO mice and analysed for chemotaxis towards the chemokine KC (A, data expressed as the % of the positive control), analysed by 2-way ANOVA, and for phagocytosis of AF488 labelled heat killed E Coli when incubated at an MOI of 1 (B). Phagocytosis was measured in cells cultured in an iced water bath ("ice control") and at 37°C to control for adhesion of bacteria to the extracellular surface of the neutrophils. (A) n=6 over 2 experiments, (B) n=8 over 2 experiments. Data represent individual data points and the mean ± SEM.
MOI; multiplicity of infection

PHD1 deficient neutrophils show enhanced respiratory burst capacity and increased oxidative stress

I went on to investigate whether inflammatory PHD1 deficient neutrophils had an altered capacity for reactive oxygen species (ROS) generation. This experiment was also carried out using whole animal $PHD1^{-/-}$ mice (KO_{PHD1}) with C57/Bl6 wild-type controls rather than the $PHD1^{fl/fl}$ MRP8Cre mouse line. This was due to the spectral overlap between the GFP tag on the MRP8Cre mouse line and the fluorescent signal associated with DCF generation.

I found no difference in baseline ROS generating capacity between genotypes (Fig 5.5A) and, although it did not reach statistical significance, there was a trend towards an increased ROS generating capacity in response to f-Met-Leu-Phe (fMLF) stimulation in the KO_{PHD1} neutrophils (Fig 5.5A). In order to define the neutrophil response to fMLF, I calculated the increase in ROS following fMLF stimulation. This showed a significantly greater response in KO than WT neutrophils (Fig 5.5B).

In order to further characterise this phenotype, I undertook HPLC-MS analysis of BAL neutrophil metabolites from WT and KO mice, 24 hours post-nebulised LPS. I found that the increased ROS generating capacity of PHD1 deficient neutrophils was associated with increased levels of metabolic oxidative stress, as measured by the ratio of NADP:NADPH, which was significantly higher in KO than WT neutrophils (Fig 5.5C). Additionally, there was a trend towards a higher proportion of oxidised glutathione (GSSG) to reduced glutathione (GSH) (Fig 5.5D).

There is no associated increase in the abundance of OxPPP intermediaries in KO neutrophils (Fig 5.5E) but as this is a snapshot of the neutrophil metabolic profile, rather than a tracing assay capable of measuring flux, these data do not exclude the possibility of increased OxPPP flux in PHD1 deficient neutrophils.

Figure 5.5

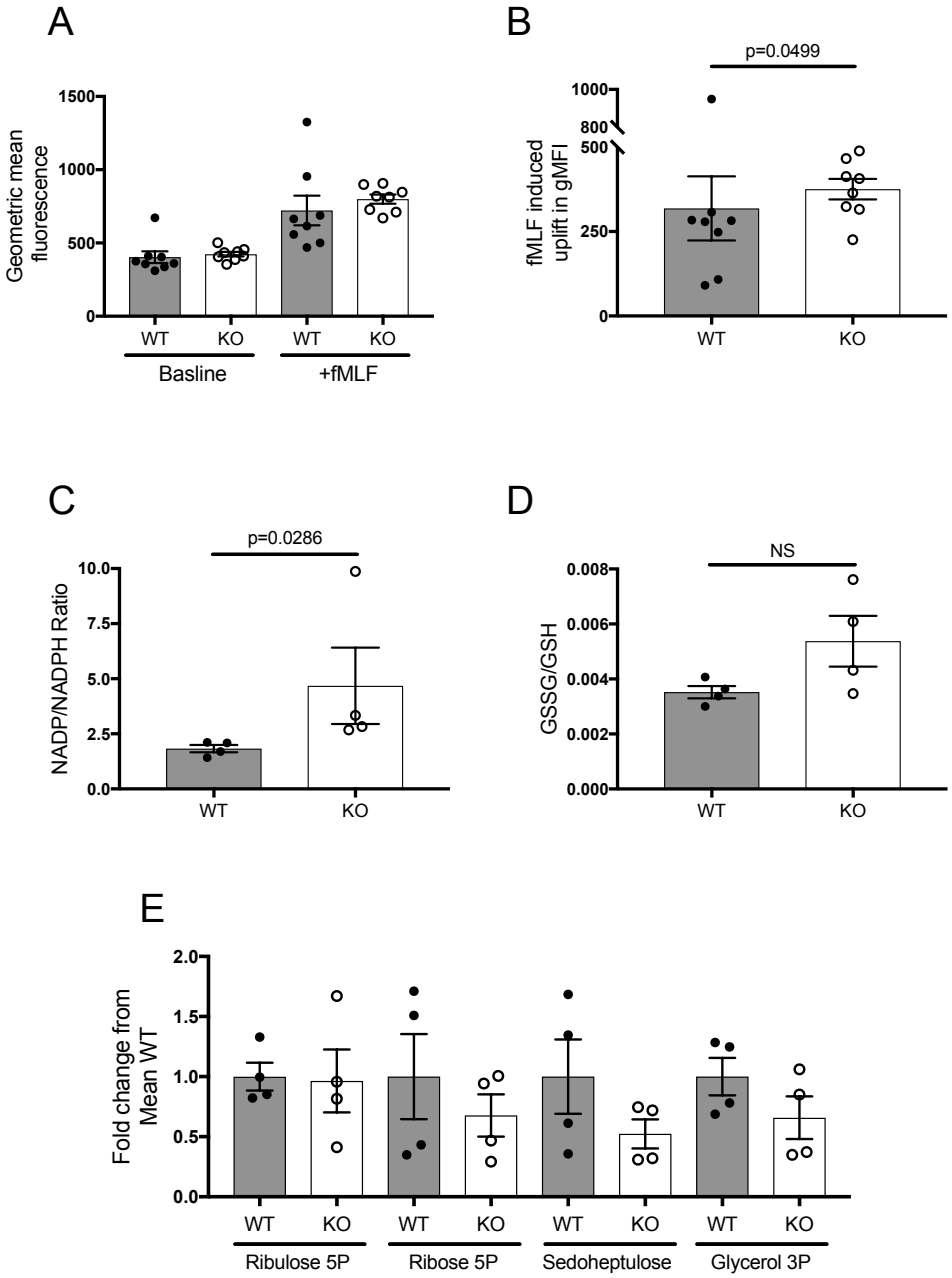


Figure 5.5: PHD1 deficient neutrophils generate more ROS and display increased oxidative stress

Measurement of WT and KO_{PHD1} BAL neutrophil ROS generation at baseline and in response to stimulation with fMLF (A). Respiratory burst activity as calculated by the uplift in DCF gMFI following fMLF stimulation (B), Mann-Whitney test of significance. HPLC-MS analysis of the ratio of NADP:NADPH (C) and the fraction of oxidised glutathione (D) in WT and KO BAL neutrophils isolated 24 hours post-LPS nebulisation, (C) & (D)Mann-Whitney test of significance. (E) Abundance of OxPPP metabolic intermediaries in WT and KO BAL neutrophils, expressed as fold change from normoxia. (A)&(B) n=8 over 2 experiments. (C)-(E) n=4. Data represent individual data points and the mean ± SEM.

fMLF; f-met-leu-phe, OxPPP; oxidative pentose phosphate pathway

PHD1 deficiency alters hypoxic neutrophil dynamics *in vivo*

In order to investigate how this phenotype of enhanced oxidative stress would affect neutrophil function *in vivo*, I utilised the LPS induced acute lung injury model in both normoxic and hypoxic conditions. There was no significant difference in the body temperatures of WT and KO mice over the 24-hour time course (Fig 5.6A & 5.6B). In normoxic conditions, there was no significant difference between genotypes in the number of neutrophils in the BAL fluid at 24 or 48 hours (Fig 5.6C) however, in hypoxia the neutrophil dynamics were altered. At 24 hours, KO mice had a significantly higher number of BAL neutrophils than their WT counterparts (Figure 5.6D). In wild-type mice housed in hypoxia, neutrophil numbers in the BAL continue to rise up to 48 hours post-LPS (Figure 5.5D). This trajectory is reversed in KO mice where neutrophil numbers fall between 24 and 48 hours and this results in a significant difference between genotypes in terms of resolution of neutrophil numbers (Fig 5.6E). These data suggest that PHD1 deficiency may result in enhanced resolution of neutrophilic inflammation but only in the context of a hypoxic challenge.

Figure 5.6

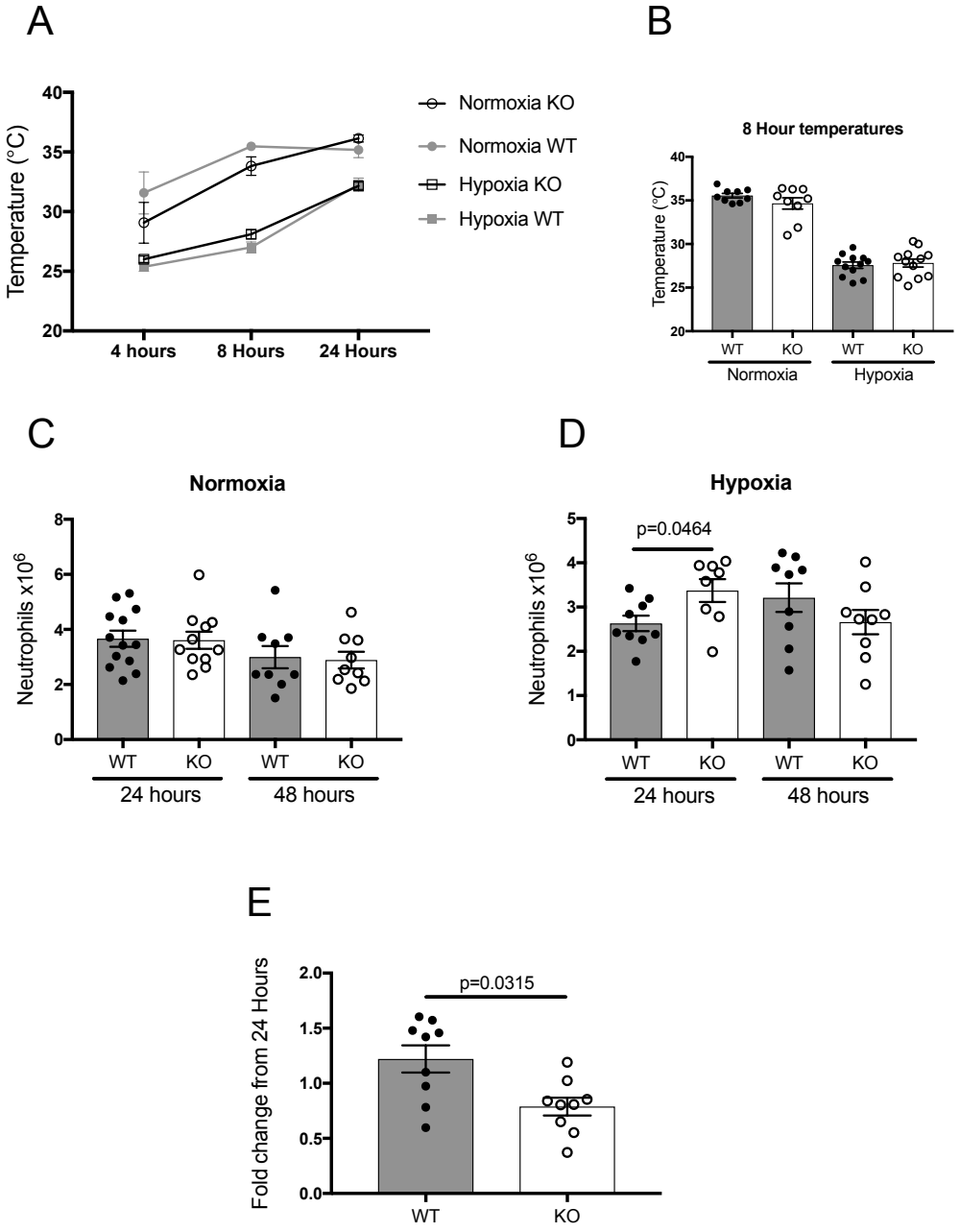


Figure 5.6: PHD1 deficiency alters neutrophil recruitment and resolution *in vivo*
Body temperatures of WT and KO mice housed in normoxia or hypoxia for 24 hours following nebulised LPS (A) n=6 over 2 experiments, 2-way ANOVA with multiple comparisons. (B) Body temperatures of WT and KO mice 8 hours after nebulised LPS housed in either normoxia or hypoxia, ordinary one-way ANOVA with multiple comparisons n≥12 over 3 experiments. BAL neutrophil counts of WT and KO mice 24 and 48 hours after nebulised LPS housed in normoxia (C) or hypoxia (D) n=8-9 over 2 experiments, Mann-Whitney test of significance. Resolution of neutrophilic inflammation in hypoxia as calculated by the fold change in neutrophil numbers at 48 hours compared to mean 24-hour counts (E) n=9 over 2 experiments, Mann-Whitney test of significance.
(A) Data represent mean ± SEM, (B)-(E) data represent individual data points and the mean ± SEM.

PHD1 deficiency does not significantly alter neutrophil driven lung damage

I went on to analyse the effect of neutrophil PHD1 deficiency on lung injury in this model. As previously described, hypoxia results in an increased level of neutrophil elastase activity in the BAL supernatant of WT mice and this pattern persisted in KO mice (Figs 5.7A&B) with no significant differences between the genotypes in either normoxia or hypoxia at 24 hours. The pro-resolution phenotype of hypoxic KO mice described in Fig 5.6 does not correlate with significantly lower BAL elastase activity in KO mice at 48 hours. I also measured BAL supernatant albumin concentration. Once again, hypoxia was associated with a sharp increase in BAL albumin levels and this was true for KO as well as WT mice at 24 hours (Figs 5.7C&D). In normoxia, there was no difference between genotypes at 48 hours. In hypoxia there was a trend towards lower albumin levels in KO mice (mean albumin concentration 1673ug/ml in KO versus 2066ug/ml in WT) but this did not reach statistical significance.

Figure 5.7

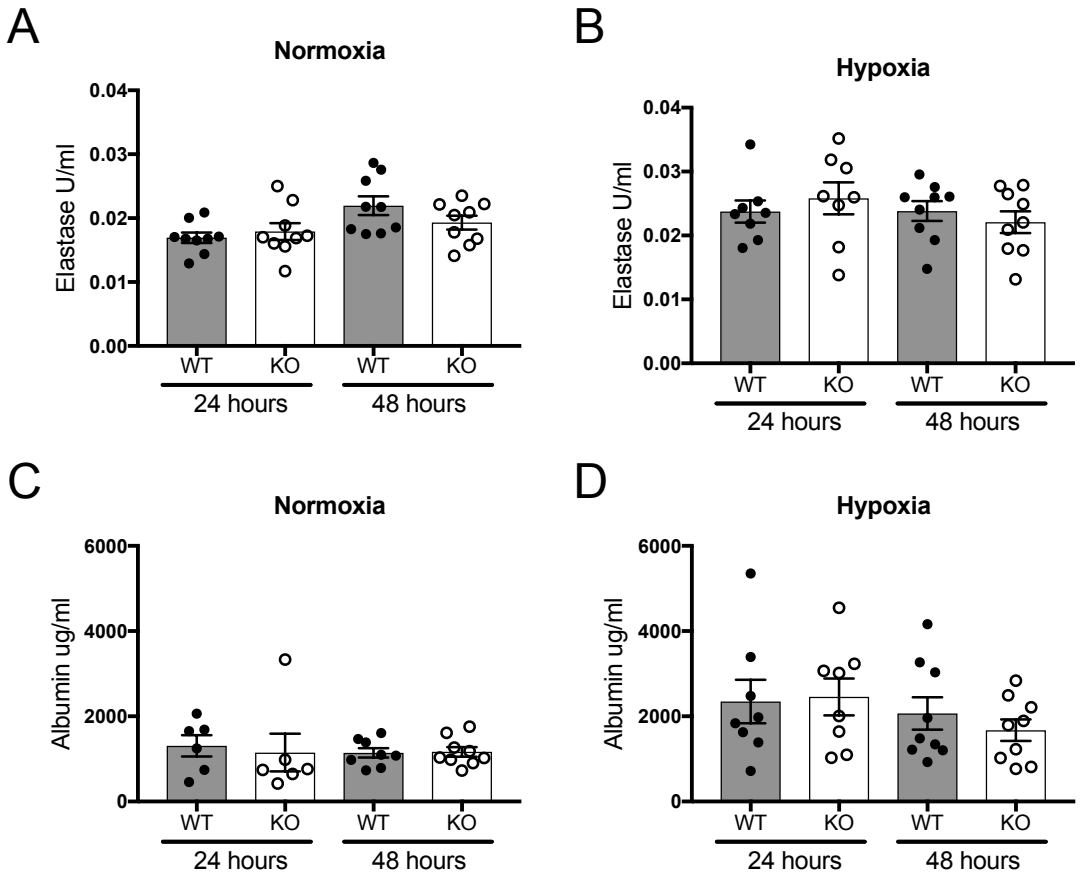


Figure 5.7: BAL elastase activity and albumin concentrations are unchanged between genotypes

Elastase activity as measured by the Elastase EnzCheck kit in BAL supernatant from WT and KO mice 24 or 48 hours after nebulised LPS housed in normoxia (A) or hypoxia (B). Albumin concentration in BAL supernatant from WT and KO mice 24 or 48 hours after nebulised LPS housed in normoxia (C) or hypoxia (D). n≥6 over 2 experiments, data represent individual data points and the mean ± SEM.

PHD1 deficiency alters neutrophil apoptosis in a context specific fashion

In order to interrogate the apparent resolution phenotype I had observed *in vivo*, I went on to investigate how PHD1 loss altered neutrophil apoptosis. I analysed blood and BAL neutrophils from WT and KO mice which had been treated with nebulised LPS and housed in normoxia for all apoptosis experiments. *Ex vivo* culture of peripheral blood neutrophils showed that, in normoxic culture, PHD1 deficiency results in significantly reduced rates of neutrophil apoptosis (Fig 5.8A). Both genotypes demonstrated reduced apoptosis in hypoxia although this did not reach statistical significance in the KO mice (p=0.08). Furthermore, in hypoxic culture, PHD1 deficiency was associated with a trend towards reduced neutrophil apoptosis in comparison to WT hypoxic neutrophils but, again, this did not reach statistical significance. Thus, in blood neutrophils, PHD1 deficiency is pro-survival but this effect is superseded by the pro-survival effect of hypoxic culture. Nevertheless, hypoxic PHD1 deficient neutrophils showed the lowest overall rates of apoptosis. This pro-survival phenotype is clearly inconsistent with the resolution of neutrophil numbers seen *in vivo* in hypoxia, so I went on to investigate whether the phenotype holds true for inflammatory lung neutrophils. BAL neutrophils were isolated 24 hours post-LPS and cultured *ex vivo* in normoxia or hypoxia. After 10 hours of culture there was no difference in the rates of apoptosis between genotypes and no evidence of enhanced survival in hypoxic culture (Fig 5.8B). After 20 hours, in normoxic culture the mean rate of apoptosis was lower in KO neutrophils but not significantly so (4.4% Vs 7.1%, p-value 0.07 by unpaired t-test). In hypoxic culture though, this effect was completely lost (Fig

5.8C) and, as was seen at 10 hours, hypoxic culture was not associated with increased neutrophil survival.

These data demonstrate that culture conditions, and the tissue compartment from which neutrophils are isolated are important determinants of neutrophil survival.

Figure 5.8

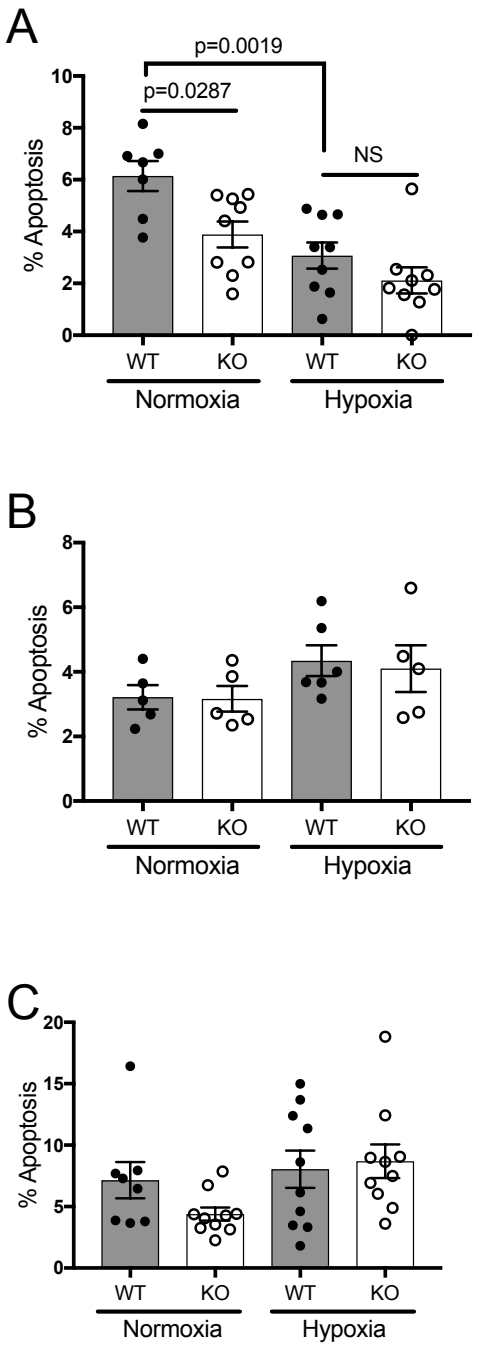


Figure 5.8: PHD1 regulates neutrophil apoptosis
Apoptosis rates of WT and KO blood (A) and BAL (B) neutrophils after 10 hours of *ex vivo* culture in either normoxia or hypoxia as analysed by nuclear morphology. Apoptosis rates of BAL neutrophils after 20 hours of *ex vivo* culture in either normoxia or hypoxia (C). n≥6 over 2 experiments, analysed by ordinary one-way ANOVA with multiple comparisons. Data represent individual data points and the mean ± SEM.

Glucose availability regulates neutrophil survival

Given the differences in apoptosis behaviour between blood and BAL neutrophils and the evidence that the lung is a glucose deplete environment, I went on to investigate the effect of glucose deprivation of neutrophil survival. Peripheral blood neutrophils from LPS-treated KO and WT mice were cultured *ex vivo* in normoxia and hypoxia in either standard RPMI or glucose free RPMI. In normoxic culture, glucose deprivation led to increased WT neutrophil survival (Fig 5.9A) on a par with the pro-survival effect of hypoxic culture. The effect was much less pronounced in KO neutrophils. Interestingly, culture of blood neutrophils in hypoxia without glucose led to such marked cell necrosis that it was not possible to analyse apoptosis rates by morphology. This suggested that, in blood neutrophils, hypoxic survival is dependent on glucose availability. Analysis of BAL neutrophils cultured *ex vivo* in the same conditions showed that glucose deficiency in normoxia appears to promote neutrophil survival (although not statistically significant in this experiment). However, unlike the peripheral blood neutrophils, BAL neutrophils can survive in hypoxic culture without glucose with similar rates of apoptosis as those seen in glucose replete hypoxic culture (Fig 5.9B). Thus, a number of physiological stresses can modulate neutrophil apoptosis, including oxygen tension (which has long been established in the literature[21,69]) and glucose availability. Furthermore, BAL neutrophils and blood neutrophils appear to respond differently to these stimuli.

Figure 5.9

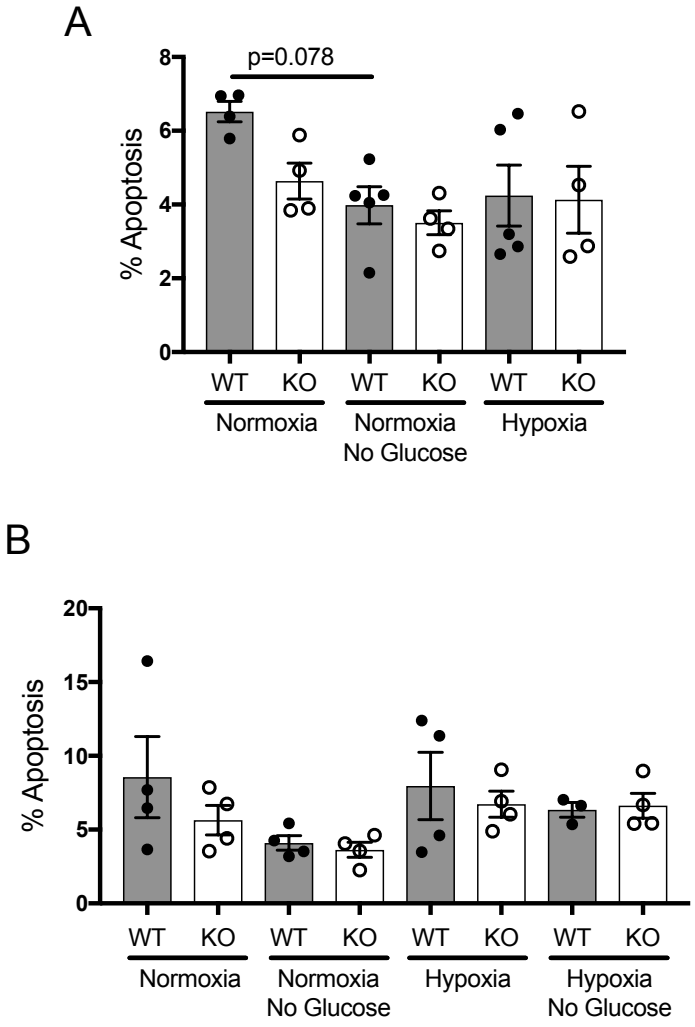


Figure 5.9: Glucose availability regulates neutrophil survival
Apoptosis rates of WT and KO neutrophils after 10 hours of *ex vivo* culture in normoxia or hypoxia ± glucose, analysed by nuclear morphology of blood neutrophils (A), n=4-5 and BAL neutrophils (B), n=4. Ordinary one-way ANOVA with multiple comparisons. Data represent individual data points and the mean ± SEM.

Hypoxia abrogates metabolic differences between PHD1 deficient and wild-type neutrophils

Analysis of the metabolic profiles of BAL neutrophils from hypoxic KO and WT mice provides some insights into the apoptosis data discussed previously. When BAL neutrophils were cultured in hypoxia and without glucose, rates of apoptosis were equivalent between genotypes, this is mirrored by unchanged abundance of glycolytic intermediaries in WT and KO BAL neutrophils isolated from hypoxic mice 24 hours after nebulised LPS (Fig 5.10A) and equivalent energy charge between genotypes (Fig 5.10B).

The data from normoxic BAL neutrophils presented in Figure 5.5C&D showed that there was increased oxidative stress in KO neutrophils. Interestingly, when these data are compared with that acquired from hypoxic neutrophils, the difference between genotypes is lost with no significant differences seen between genotypes in NADP/NADPH or GSSG/GSH in the samples from hypoxic animals (Fig 5.10C&D). Furthermore, the values found in hypoxia for both genotypes are more similar to those seen in normoxic KO samples. This raises the possibility that, at baseline, PHD1 deficient neutrophils have a pseudo-hypoxic metabolic phenotype. To further investigate this possibility, I measured HIF-1α levels using a fluorescently labelled antibody and flow cytometry to measure staining. In this pilot experiment, there was a small increase in the mean fluorescence of KO compared to WT BAL neutrophils from normoxic mice (Fig 5.10E). This observation would be consistent with the enhanced survival seen in normoxic PHD1 deficient neutrophils but there are some important caveats. As shown in Figures 5.6 and 5.7, the *in vivo*

phenotype of PHD1 deficiency in normoxia does not represent a phenocopy of hypoxic challenge as described in chapter 3 of this thesis, suggesting that there are fundamental differences between a hypoxic neutrophil and a PHD1 deficient neutrophil. Additionally, PHD1 deficient BAL neutrophils respond to glucose deprivation in the same way as WT neutrophils in terms of apoptosis whereas hypoxic blood neutrophils appear to respond very differently. Thus, while some elements of the PHD1 KO phenotype are consistent with a 'HIF-stabilised' neutrophil signature, the context is key in determining outcome. Importantly, *in vivo* hypoxic challenge seems to overcome the metabolic PHD1 KO signature but also alter neutrophil dynamics *in vivo*. These data did raise interesting questions regarding the role of glucose in regulating neutrophil survival and I went on to investigate this in greater detail.

Figure 5.10

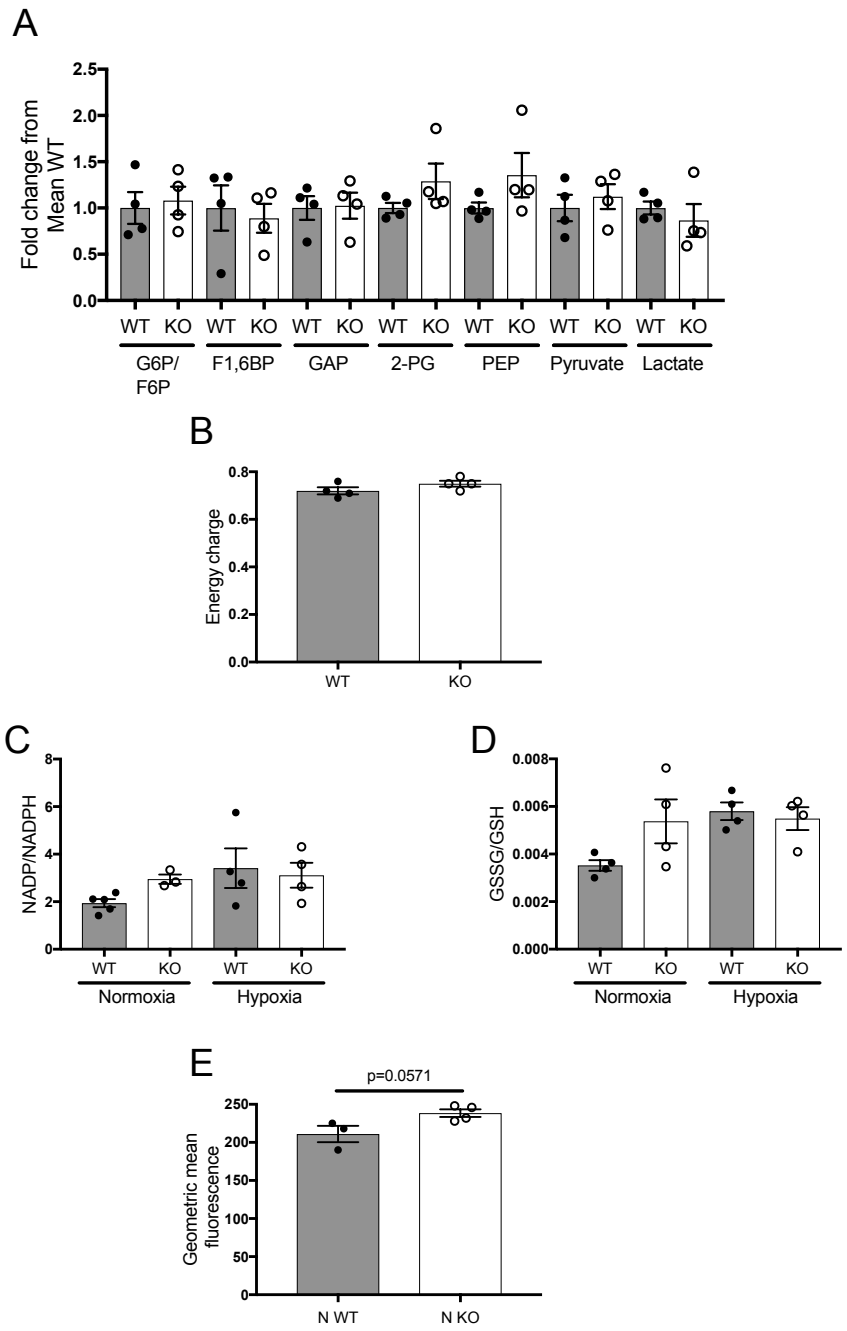


Figure 5.10: Hypoxia mitigates differences in PHD1 deficient neutrophil metabolism
(A) HPLC-MS analysis of metabolic intermediaries of glycolysis in WT and KO neutrophils from hypoxic mice expressed as fold change from the mean normoxic value. Analysed by multiple t-tests corrected for multiple comparisons by the Bonferroni method. (B) Energy charge of BAL neutrophils isolated from hypoxic WT and KO mice 24 hours post LPS. HPLC-MS analysis of the ratio of NADP:NADPH (C) and the fraction of oxidised glutathione (D) in WT and KO BAL neutrophils isolated 24 hours post-LPS nebulisation from both normoxic and hypoxic mice. (E) HIF-1α quantity measured by intracellular staining and flow cytometry of BAL neutrophils from normoxic WT and KO mice 24 hours post-LPS, Mann-Whitney test of significance. (A)-(D) n=4, (E) n=3-4. Data represent individual data points and the mean $\pm$ SEM.
G6P/F6P;glucose or fructose-6-phosphate, F-1,6-BP; fructose-1,6-bisphosphate, GAP; glyceraldehyde-3-phosphate, 2-PG; 2-phosphoglycerate, PEP; phosphoenolpyruvate.

Albumin availability modulates the impact of glucose deprivation on neutrophil survival

To try to understand the mechanisms underlying the role of glucose in regulating neutrophil apoptosis, I used peripheral blood neutrophils from healthy human volunteers. I found that, as was observed in murine blood neutrophils, glucose deprivation in normoxic culture has a pro-survival effect (Fig 5.11A) in neutrophils cultured for 20 hours but in hypoxic culture, glucose deprivation has a pro-apoptotic effect (Fig 5.11B). This subtle effect on apoptosis in hypoxic glucose free culture was not consistent with the markedly necrotic morphology I observed in murine blood neutrophils and so I also analysed cell numbers following 20 hours of culture. This showed a profound cell loss in hypoxic glucose free culture with approximately an 80% reduction in cells numbers over 20 hours (from $5x10^6$/ml to $0.7x10^6$/ml) (Fig 5.11C).

The data in Figure 5.9B does suggest that BAL neutrophils are protected from the detrimental effect of glucose deprivation in hypoxia. There are a number of potential reasons for this: the process of transmigration is likely to alter the identity of the neutrophil, BAL neutrophils will have been exposed to an inflammatory milieu with a number of pro-survival elements, including GM-CSF, TNF-α and other cytokines. Finally, the availability of other sources of energy, including extracellular proteins, may also be protective during glucose depletion. It is likely that these factors act in combination to promote neutrophil survival. I was interested to see if supplementing glucose free media with albumin (in the form of BSA) could provide any protection in hypoxic glucose free culture. Supplementing the glucose deplete media with 2% BSA did not

result in different rates of apoptosis in hypoxia (Fig 5.11D) but did result in a significant increase in the remaining cell numbers at 20 hours (Fig 5.11E). Although this improvement was small and did not return the cell number back to levels seen in glucose replete media, these data do suggest that protein can, at least to some extent, replace glucose as a substrate for neutrophils, consistent with the labelling data presented in Figure 4.13.

Figure 5.11

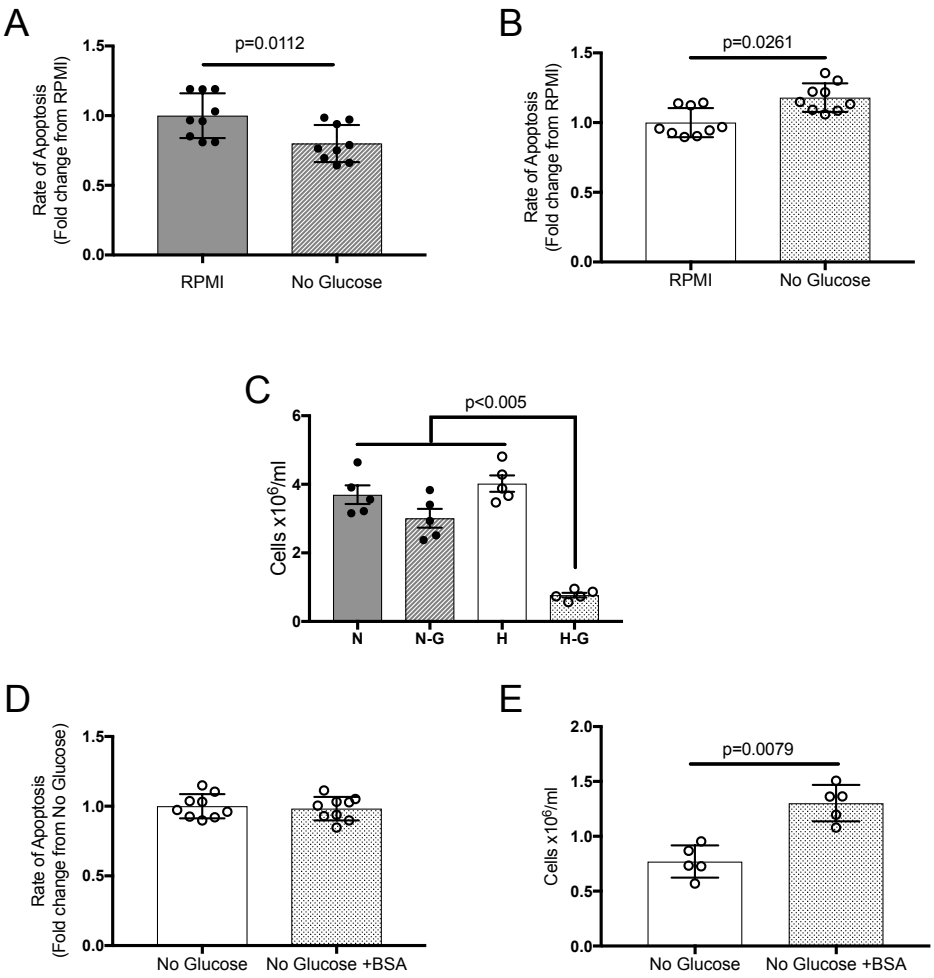


Figure 5.11: Albumin availability partially compensates for glucose deprivation in *in vitro* culture

Rates of apoptosis as measured by flow cytometry in human peripheral blood neutrophils after 20 hours of culture in media ± glucose in normoxia (A) or hypoxia (B) n=9 over 4 experiments, (A)&(B) analysed by a single one-way ANOVA with multiple comparisons. Neutrophil counts measured using CountBright beads and flow cytometry following 20 hours of culture in normoxia or hypoxia ± glucose (C), n=5 over 3 experiments, analysed by row-matched one-way ANOVA with multiple comparisons. The initial concentration of neutrophils at T_0 was $5x10^6$/ml. Rates of apoptosis (D) and residual neutrophils counts (E) of human peripheral blood neutrophils cultured for 20 hours in hypoxia without glucose ± 2% BSA supplementation, (D) n=9 over 4 experiments, (E) n=5 over 3 experiments, Mann-Whitney test of significance. Data represent individual data points and the mean ± SEM.

Discussion

Our group have previously identified specific roles for PHD2[76] and PHD3[77] in regulating neutrophil inflammatory responses. The data in this chapter demonstrate that neutrophil specific PHD1 deficiency can alter neutrophil dynamics during an inflammatory response but that this is highly context dependent. I have characterised a new mouse line, generated using Cre-Lox technology. PHD1 floxed mice were developed by our collaborators in Leuven, Belgium and Oxford, UK. We have crossed them with the MRP8 driven Cre line to generate neutrophil specific PHD1 knock-down which is evident in bone marrow, blood and lung neutrophils. Loss of PHD1 does not alter the expression of the other PHD isoforms at a transcriptional level. This is consistent with other models of cell specific, isoform specific, PHD knockdown. The failure of PHD isoforms to compensate for loss of one another in mammals is strongly suggestive of specific roles for each isoform without major redundancy within the system.

Neutrophil specific PHD1 deficiency does not alter the make-up of the circulating leucocyte populations at baseline or in response to an LPS challenge. PHD1 deficient neutrophils carry out normal chemotaxis and phagocytosis. Importantly, these *ex vivo* studies of neutrophil function were only carried out on neutrophils from normoxic mice and in the future, it will be important to investigate how both *in vivo* and *ex vivo* hypoxia may augment the effect of PHD1 deficiency.

Data from other cell types has consistently shown that PHD1 deficiency can result in altered metabolic phenotypes, particularly in the setting of ischaemic

injury. In skeletal muscle, the primary metabolic adaptation was a reduction in oxidative stress due to a shift from oxidative phosphorylation to the anaerobic process of glycolysis and therefore preserved ATP generation, despite oxygen deprivation[204]. In cerebral ischaemia PHD1 deficiency is also protective but the metabolic mechanism is distinct. PHD1 deficient neurons displayed reduced glycolysis and glucose consumption but increased glutamine oxidation and, critically, increased redox buffering capacity. This was due to enhanced flux through the oxidative pentose phosphate pathway (OxPPP), increased NADPH generation and therefore replenishment of reduced glutathione[207]. We were interested to investigate how PHD1 deficiency might regulate these metabolic pathways in neutrophils because, as has been discussed previously, they are predominantly glycolytic at baseline so a 'switch' from oxidative phosphorylation cannot occur. Additionally, while neurons may use OxPPP to generate redox buffering capacity, neutrophils use NADPH to drive ROS generation via the action of the NADPH oxidase and so increased flux through this pathway might be predicted to alter neutrophil inflammatory function rather than redox potential.

PHD1 deficient neutrophils demonstrate enhanced ROS generation in response to fMLF and this was associated with an increase in the ratio of NADP:NADPH and a higher fraction of oxidised glutathione as shown in Figure 5.5. This increased oxidative stress is therefore in contrast to the response described in neurons. The mechanism which underlies this increased ROS production in PHD1 deficient neutrophils requires further investigation. In neurons this is regulated in part by the enzyme TIGAR which has fructose-2,6-

bisphosphatase activity and thus directs glucose away from glycolysis and into OxPPP. In skeletal muscle the metabolic switch is regulated by pyruvate dehydrogenase kinase upregulation which blocks the entry of pyruvate into the TCA cycle. Analysing expression or activity of these regulatory metabolic enzymes will therefore be of interest in clarifying the metabolic consequences of PHD1 loss in neutrophils.

The *in vivo* data presented in this chapter suggest that in normoxia, neutrophil specific PHD1 deficiency does not alter the outcome of an inflammatory insult. However, concurrent hypoxic challenge does lead to differences in neutrophil recruitment and resolution between genotypes. Whereas in wild-type mice, neutrophilic inflammation persists at 48 hours, PHD1 deficiency results in enhanced resolution of neutrophil numbers in hypoxia at 48 hours. This is suggestive of a highly context specific role for PHD1 in hypoxic neutrophils and is in contrast to findings in PHD2 deficient neutrophils which resulted in enhanced neutrophil survival and prolonged neutrophilic inflammation in a normoxic acute lung injury model[76]. Both PHD1 and PHD2 deficiency result in enhanced survival of cultured blood neutrophils in normoxia. In PHD2 deficient neutrophils this difference persists in hypoxic culture whereas the data in this chapter show that PHD1 deficiency does not significantly enhance neutrophil survival above and beyond the increase caused by hypoxia. This observation led us to investigate the apoptosis phenotype of PHD1 deficient neutrophils in more detail and, although these experiments did not fully explain the *in vivo* findings, they did identify the availability of glucose as a novel regulator of neutrophil survival. Firstly, we found that BAL and blood

neutrophils are differentially regulated by both hypoxia and glucose availability. Peripheral blood neutrophils display enhanced survival in hypoxia, as has been established in the literature for many years[21,69]. However, this is not true for inflammatory BAL neutrophils whose survival *ex vivo* is unchanged in hypoxia. In contrast, hypoxic BAL neutrophils are tolerant of glucose depletion while hypoxic blood neutrophils display increased apoptosis and profound cell loss in these conditions. These data are consistent with the findings in chapter 4 which suggest that hypoxia and glucose availability have the capacity to alter neutrophil signalling pathways such as the mTOR pathway and are important in regulating catabolism of extracellular proteins in neutrophils.

The disastrous consequences for blood neutrophils of glucose deprivation in hypoxia are partially rescued by the addition of albumin to the culture media. Albumin is equally abundant in the BAL fluid of PHD1 deficient mice and WT mice in hypoxia (as shown in Figure 3.7D) and so substrate availability is not limiting the persistence of these cells. However, it is possible that PHD1 deficient neutrophils are not able to utilise extracellular proteins to same extent as their wild-type counterparts and consequently cannot persist in the low glucose environment of the hypoxic airway. Ordinarily, hypoxia would inhibit mTORC1 activity through increased AMPK activity and increased REDD1 activity. If this hypoxic down-regulation of mTORC activity did not occur, then the subsequent upregulation of lysosomal protein catabolism would be prevented. When considering the consequence of genetic ablation (rather than acute knock-down) of one of the oxygen-sensing PHD enzymes, the duration, as well as the amplitude, of hypoxic responses are important to consider.

Hypoxic preconditioning is an established phenomenon in multiple systems including in neutrophilic inflammation. Our group has previously demonstrated that prolonged exposure to hypoxia prior to an infectious insult abrogates the deleterious effect of acute hypoxia[143]. It is interesting to consider whether PHD1 deficiency leads to increased HIF activity at baseline and as a consequence 'resets' the HIF threshold. The pro-survival phenotype observed in peripheral blood neutrophils is consistent with this, as is the increased oxidative stress which is on a par with that seen in hypoxic WT neutrophils. If this chronic baseline increase in HIF activity were to act as a preconditioning stimulus, one would hypothesis that PHD1 deficient neutrophils may fail to respond maximally to a second hypoxic challenge. One potential consequence of this in the lung is a failure of the hypoxic upregulation of catabolic pathways discussed in the previous data chapters. If PHD1 deficient neutrophils do not suppress mTORC and upregulate protein catabolism they may not be able to persist as WT neutrophils do in the inflamed lung, consistent with the more rapid resolution of cell numbers seen in hypoxic KO mice at 48 hours.

Further work is needed to test this hypothesis. Measurement of HIF-1α and HIF-2α levels (and indeed transcription of HIF target genes) in blood and lung neutrophils will be key in determining if PHD1 deficiency does result in enhanced HIF activity and pseudo-hypoxia. It will also be important to assess how PHD1 loss alters the dynamics of HIF stabilisation in response to a hypoxic challenge. In the literature PHD1 phenotypes are variably associated with HIF activity. The skeletal muscle phenotype describes by Aragones et al was dependent on HIF-2α activity[204]. HIF-2α was also required for

protection in a liver ischaemia-reperfusion model in PHD1 deficient mice[206]. Conversely, in the model of cerebral ischaemia described by Quaegebeur et al, the protection conferred by PHD1 loss was independent of HIF1-α, HIF-2α and HIF-1β[207] but was dependent on NF-κB activity.

In order to test the theory that PHD1 deficient neutrophils cannot utilise extracellular protein to the same extent as hypoxic WT neutrophils, measurement of mTORC activity and lysosomal function should be carried out. Although this further work is required to establish the full consequences of PHD1 loss on neutrophil function, it is a potentially appealing phenotype in lung inflammation. PHD1 deficient neutrophils appear to be functionally competent but do not persist in the damaging way that hypoxic WT neutrophils do. It will be important to investigate the consequences of PHD1 loss in infection models, as well as the sterile inflammatory insult described here to ensure that PHD1 deficient neutrophils can adequately control infection. Their preserved chemotaxis, and phagocytosis, coupled with their enhanced respiratory burst is strongly suggestive that they will be effective bactericidal cells. This raises the possibility that inhibition of PHD1 activity may facilitate a functional innate immune response with a rapid 'off switch' to avoid dysfunctional inflammation.

Chapter 6: Discussion and Future Directions

Summary of key findings

Systemic hypoxia frequently complicates respiratory disease. Additionally, inflammatory insults in many organs may be associated with localised hypoxia. The regulation of inflammation by hypoxia is therefore of importance in understanding, and therefore preventing, dysfunctional inflammation. We have previously shown that, in both pulmonary and skin infections, concurrent exposure to hypoxia results in a significant increase in neutrophil mediated morbidity and mortality[143]. The overall aim of this project was to interrogate the mechanisms which underlie this phenotype.

Hypoxic neutrophils are hyperinflammatory

I have shown that hypoxia represents a damaging 'second hit' in sterile neutrophilic inflammation with evidence of systemic illness and localised neutrophilic injury in the lung. I used proteomic analysis of inflammatory lung neutrophils to investigate what drives this damage. This analysis confirmed our hypothesis that hypoxia dramatically alters the phenotype of a neutrophil. Proteomic analysis provides important insights into cellular identity: it is an unbiased approach, which therefore has inherent advantages in terms of identifying novel therapeutic targets. It also provides a snapshot of the pathways and processes which are active and important in the cells of interest because it provides quantitative information about which proteins are expressed and, crucially, how the abundance of these proteins is regulated by a given stimulus. Although in this dataset we did not analyse post-translational modifications in a proteomic dataset, this is an emerging technique which can

provide even more detailed information about the identity and function of proteins within a population of cells.

Several of the experiments which are detailed in this thesis illustrate the importance of the microenvironment, including the tissue origin (i.e. blood versus BAL) in the regulation of neutrophil function. These differences are likely to be multifactorial: the process of transmigration from the circulation fundamentally alters the phenotype of a neutrophil, tissue neutrophils are exposed to different inflammatory stimuli including cytokines, DAMPs and pathogens, and, finally, the metabolic environment in the tissue is distinct from the circulation. I have shown that the lung represents a low glucose, high protein environment and that this has important consequences for infiltrating neutrophils. Recapitulating these stimuli *in vitro* is challenging and so we focussed on neutrophils isolated from the site of inflammation. To our knowledge, this is the first proteomic analysis of inflammatory tissue neutrophils and, for the reasons detailed above, it was important to use this population, rather than blood or bone marrow neutrophils, to understand the *in vivo* phenotype which we observed.

In keeping with the sickness and lung damage we observed in hypoxic mice, hypoxic neutrophils were found to have a hyperinflammatory phenotype with wide-ranging adaptations. Hypoxic neutrophils alter their cell surface receptor expression resulting in an enhanced capacity to respond to inflammatory stimuli such as TNF-α, formylated peptides and GM-CSF. These factors are also established survival stimuli for neutrophils and so this cell surface repertoire is likely to contribute to persistent, as well as enhanced,

inflammation. While the majority of the work presented here has focussed on the regulation of neutrophil function specifically by hypoxia, this response does not occur in isolation. Hypoxia is likely to alter the behaviour of other immune cells and to influence other cell types, particularly the endothelium and epithelium within the lung tissue. All of these factors combined generate a profoundly inflammatory niche in which the hypoxic neutrophils operate, resulting in a vicious cycle of inflammation, neutrophil recruitment and further tissue damage leading to persistent neutrophilic inflammation.

Hypoxia has previously been found to enhance neutrophil degranulation *in vitro*[142] and we provide evidence here of enhanced hypoxic degranulation *in vivo*. It was therefore surprising that this enhanced degranulation did not correspond to lower levels of intracellular granule proteins in the proteome of hypoxic neutrophils. These findings were strongly suggestive of continued synthesis of inflammatory granule proteases by lung neutrophils. This is in contrast to our current understanding of neutrophil biology which suggests that granule proteins are synthesised only during neutrophil maturation in the bone marrow and that the inflammatory capability of the neutrophil is limited to releasing these proteases from their preformed granules. Once this had occurred, the neutrophil was thought to be without capacity to regenerate inflammatory granule proteases. However, the persistent expression of mRNA coding for granule proteins is consistent with the ongoing synthesis of these proteins by tissue neutrophils, given the right conditions. Furthermore, *ex vivo* culture of BAL neutrophils with ^{13}C and ^{15}N labelled amino acids has allowed us to identify those proteins which are synthesised by these tissue neutrophils.

These data identified incorporation of labelled amino acids into the most abundant proteins, which is to be expected. However, we also identified newly synthesised, labelled granule proteins including lysozyme C2 and neutrophil granule protein (NGP), consistent with ongoing synthesis of granule proteins by inflammatory neutrophils. This ongoing synthetic capacity is a novel finding and enhances our understanding of how neutrophilic inflammation may become persistent and dysfunctional in the lung. Hypoxic neutrophils therefore have an enhanced capacity for inflammation and I next sought to understand how this is fuelled.

Extracellular proteins fuel hypoxic neutrophils

Protein-rich exudates are pathognomonic of acute lung injury. I have shown here that, not only is protein leak a marker of lung damage, but it has the capacity to drive further inflammation. I have shown for the first time that neutrophils take up and break down proteins from the environment and, using labelled protein lysates, I have shown that the glutamine from scavenged proteins can be metabolised by neutrophils to provide substrate for central carbon metabolism. Work from other members of our group has shown that neutrophils can utilise glutamine for central carbon metabolism (Sadiku et al 2018, under review) and here we show that proteins are a source of this substrate. The identification of this pathway is also supportive of the hypothesis that amino acids from scavenged proteins may further contribute to inflammation by providing substrate for de novo synthesis of inflammatory mediators, including granule proteins. This is particularly relevant in the hypoxic lung where protein leak is high, but this process is likely to be relevant

in other inflammatory and infective contexts too. Neutrophils have been established to contribute to the initiation and propagation of joint inflammation in rheumatoid arthritis (RA) [213]. The rheumatoid joint is a relatively hypoxic environment, Ng et al[214] measured oxygenation *in vivo* and found a mean oxygen tension of approximately 3%. Furthermore, the degree of hypoxia was found to correlate with the magnitude of the inflammatory response, with higher concentrations of IL-1β, TNF-α and IFN-γ[214]. In keeping with the changes found in lung inflammation, alterations in microvascular permeability are found within the inflamed joint, resulting in protein leak into this space. Indeed, when compared to osteoarthritis, rheumatic joints were found to have significantly higher flux of plasma proteins, including albumin[215]. In health, glucose levels in the joint are similar to those in the plasma but in rheumatoid arthritis glucose levels may be low[216]. Thus, the rheumatic joint represents a similar, low glucose, high protein niche to the inflamed lung and it is tempting to speculate that neutrophils at this site can also exploit their environment to fuel inflammation.

Chloroquine attenuate LPS induced acute lung injury

Further evidence of the importance of this pathway is provided by the chloroquine treatment of mice with acute lung injury. I first examined the capacity of chloroquine to inhibit neutrophil protein breakdown *in vitro* and then trialled *in vivo* administration of this lysosomal inhibitor. Although chloroquine may alter neutrophil recruitment when given early in the inflammatory process, delayed administration does not significantly change neutrophil numbers in the LPS-induced acute lung injury. However, intraperitoneal injection of

chloroquine does result in a reduction in the capacity of BAL neutrophils to breakdown proteins (as measured by DQ-Green albumin fluorescence) and, importantly, in reduced elastase activity in the BAL supernatant and improvement in hypothermia. In this proof of concept experiment, I did not see such a marked improvement in these parameters in hypoxic mice, although there was a subtle shift towards improvement. It is possible that the degree of injury in hypoxia is too great to overcome using this strategy but, as discussed previously, it is also possible that the cardiovascular compromise caused by acute hypoxia resulted in less efficient absorption of the drug. A number of factors make chloroquine an attractive therapy: it has been used for decades in other diseases and as such, extensive safety data exists for its use and it is affordable. Additionally, it appears to be beneficial after the onset of inflammation and so, because development of ARDS can be difficult to predict in patients, a therapy which is efficacious after delayed administration is essential. An important consideration in developing therapies for lung disease is the potential for topical treatment in the form of inhaled or nebulised drugs. This was illustrated by a recent paper utilising pre-treatment with a nebulised TNF receptor inhibitor in the treatment of acute lung injury[203]. To our knowledge, chloroquine has never been used topically, in the lung or elsewhere. However, the potential for improved local delivery and reduced overall systemic dosing mean that this route is worthy of further consideration.

Microenvironmental regulation of neutrophil function

A recurrent theme in the data presented here is the importance of the environment in determining neutrophil fate and function. Neutrophils have long

been considered a simple, homogeneous population of cells with limited plasticity. Increasingly the literature points to a much more complex and diverse population with the capacity to critically alter inflammatory, infective and malignant processes. The identification of this functional diversity opens up new possibilities for targeting harmful neutrophil behaviour without obliterating their essential innate immune functions.

Experimental hypoxia has two roles in this work. The first is that it is a physiologically relevant stimulus due to the requirement for neutrophils to function in low oxygen environments. Secondly, it is an experimental tool, used to potentiate neutrophilic inflammation and, in this context has led to the identification of novel neutrophil behaviours which are likely to be relevant to both normoxic and hypoxic cells.

It is also important to consider that both the HIF transcription factors, and their regulatory prolyl hydroxylases may be regulated by both hypoxic and non-hypoxic stimuli, including inflammatory mediators. Once again, the context here is key. This is illustrated by the data regarding loss of PHD1 in neutrophils. Despite evidence that PHD2 is the dominant regulator of HIF under normoxic conditions[66], specific loss of PHD1 results in changes in neutrophil apoptosis in normoxic culture, an effect which is supplanted by hypoxic culture. This observation led us to consider whether PHD1 deficient neutrophils may exhibit a ‘pseudo-hypoxic’ phenotype. Further experimental work is required to investigate whether loss of PHD1 can alter basal levels of HIFα isoforms and how this may be regulated by hypoxic challenge. Importantly, this pro-survival effect of PHD1 deficiency is also lost when BAL neutrophils are examined,

irrespective of oxygen tension. It was therefore interesting to find that, *in vivo*, PHD1 loss results in more rapid resolution of hypoxic neutrophilic inflammation. This suggests that, despite the baseline pseudo-hypoxic, pro-survival phenotype, PHD1 deficient neutrophils *in vivo* did not replicate WT hypoxic neutrophil behaviour.

A number of checks and balances have been identified in the hypoxic signalling pathways discussed here, including negative feedback loops and temporal regulation of different pathway members. Chronic hypoxia represents a very different insult to acute hypoxia. Our group has found that hypoxic preconditioning prior to infection results in protection from the morbidity and mortality associated with subsequent acute hypoxia[143] and that this protection was associated with lower HIF stabilisation in preconditioned animals. Thus, chronic hypoxia appears to reset the HIF threshold, altering the responsiveness of the system to subsequent hypoxia. The PHD1 deficient neutrophils display some parallels with these data and this observation suggests that there is potential therapeutic utility in PHD1 inhibition.

Hypoxia, inflammation and metabolism are inextricably linked

The data in this thesis have also confirmed the importance of neutrophil metabolism and energetics in regulating function and therefore inflammatory outcomes. In keeping with this observation is the finding that mTOR plays a role in directing neutrophil metabolic processes. Fascinating links exist between hypoxic signalling pathways, metabolic sensing pathways (including regulation of mTOR and AMPK) and inflammation. This complex network of signals is crucial for cells to sense and respond to their metabolic environment.

A direct link between PHD activity and mTOR was recently identified in tumour cells and this paper also highlighted the role of post-translational modifications in altering activity of key enzymes including PHDs[217]. The authors identified phosphorylation of serine residue 125 (Ser125) in PHD2 as a regulatory post-translational modification. This residue was phosphorylated by the mTOR target S6 kinase (S6K) and dephosphorylated (resulting in reduced PHD2 activity) by the phosphatase PP2A, which also dephosphorylates S6K. In hypoxic tumour cells, the balance of mTOR and PP2A activity shifted in favour of PP2A resulting in reduced S6K activity and reduced PHD2 activity due to lower rates of Ser125 phosphorylation. This in turn led to enhanced survival of tumour cells due to activation of autophagy pathways.

As discussed in results chapter 2, mTOR signalling is closely link to AMP activated protein kinase (AMPK) and the balance of activity of these pathways is important in regulation of cellular energetics. A recent study of AMPK in neutrophils provided insights into the role of this kinase, particularly in the regulation of neutrophil survival and responses to hypoxia. Interestingly the authors found that in AMPK deficient neutrophils, there was a blunted response to hypoxia, as analysed by proteomic analysis of hypoxic bone marrow neutrophils from AMPK wild-type and AMPK knockout mice. This was associated with altered expression of pro-and anti-apoptotic factors in AMPK knockout neutrophils and evidence of enhanced resolution of neutrophilic infiltration in a model of limb ischaemia. This paper clearly illustrates that AMPK has a role in neutrophil function. It will be important to assess this in alternative culture conditions (including low glucose media) and, as discussed

above, to analyse neutrophils from both the blood and inflammatory tissues to elucidate more fully the role of AMPK in the regulation of neutrophil functions.

Future Directions

The work presented here highlights a number of areas which are worthy of further investigation.

Direct evidence that amino acids from scavenged proteins can be incorporated into newly synthesised proteins will be important in confirming this hypothesis which is based on indirect evidence at present. I plan to use proteins isolated from HEK cells which have been cultured with labelled Lysine and Arginine as a source of amino acids. I will culture *ex vivo* neutrophils with the labelled proteins (purified to ensure no free labelled amino acids are present) and then perform proteomic analysis to identify which, if any, newly synthesised proteins have incorporated the amino acids which originated in exogenous proteins.

I am interested in further delineating the role of mTOR in neutrophils, specifically with regard to how this kinase may direct neutrophil protein consumption and catabolism, energetics and ultimately survival. The data presented here demonstrate that mTOR is regulated by both hypoxia and glucose availability, but further work will focus on the relative importance of mTORC1 versus mTORC2 and on the downstream effects of activation and inhibition of these complexes. Additionally, the role of mTOR in directing protein translation has not been studied in neutrophils. While the data in this thesis support the hypothesis the mTOR blockade increases the availability of amino acids through enhanced protein breakdown, this would normally also be associated with reduced translation due to reduced activity of the eIF family of translational initiators. This question could be addressed by comparison of

the transcriptome and proteome in neutrophils where the mTOR pathway has been manipulated.

The finding that glucose is required for hypoxic survival of neutrophils raises a number of interesting questions requiring further study, particularly because of the relatively subtle changes in apoptosis compared to the dramatic cell loss, suggesting significant necrosis of neutrophils. Apoptosis is an energy requiring process and, as such, the glucose free hypoxia phenotype is in keeping with a complete energetic failure in these cells. Analysis of activation of AMPK and of AMP and ATP levels in these conditions will provide some insights into this phenotype. I hypothesise that this regulation is augmented in inflammatory cells, such as those isolated from the BAL fluid and this augmentation will account for some of the differences in apoptosis seen between blood and BAL neutrophils. The finding that albumin can partially improve hypoxic glucose free neutrophil persistence is consistent with the metabolic data showing that proteins can replenish central carbon metabolism, as has been shown in tumour cells. Whether this can in turn restore a favourable energetic profile, potentially through regulation of AMPK activity in this context will also be interesting to investigate.

Our understanding of the role of the prolyl hydroxylases in regulating metabolic signalling pathways in neutrophils remains incomplete. The data from Di Conza et al[217] demonstrate that these pathways are linked at multiple levels. Establishing the effect of PHD1 loss on levels of both HIF-1α and HIF-2α and investigating how HIF dynamics are altered in PHD1 deficient neutrophils during hypoxia will be important in unpicking the *in vitro* and *in vivo* data

presented here. It will also be interesting to establish whether PHD1 deficiency does alter lysosomal protein breakdown which is usually upregulated in hypoxia and whether this, in turn, is due to alterations in mTOR responsiveness.

While the literature regarding mTOR and AMPK in other cell types is extensive, it is important to look specifically at neutrophils due their unusual metabolic phenotype, their lack of capacity to replicate and their specific response to hypoxia, in particular, their prolonged survival.

Conclusions

The data presented in this thesis show that hypoxia, and hypoxic signalling pathways are critical regulators of neutrophilic inflammation. Within the hypoxic lung, a vicious cycle of degranulation, lung damage, protein leak and increased substrate availability represents a perfect storm for dysfunctional and persistent neutrophilic inflammation. These findings have led to the identification of novel therapeutic targets, such as inhibition of lysosomal function with chloroquine. Future studies will focus on the regulation of these processes by metabolic and energetic signalling pathways including those regulated by mTOR and AMPK.

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Appendix

Appendix 1: Percoll Preparations

Human peripheral blood leucocyte preparation

Percoll Concentration	For 10ml (3 gradients)	
	90% Percoll	1X PBS
73%	8.1ml	1.9ml
61%	6.8ml	3.2ml
49%	5.5ml	4.5ml

Mouse BAL neutrophil purification

Percoll Concentration	For 10ml (3 gradients)	
	90% Percoll	1X PBS
78%	8.7ml	1.3ml
69%	7.7ml	2.3ml
52%	5.8ml	4.2ml

Mouse Bone Marrow leucocyte preparation

1xHBSS+0.2% BSA:
5ml 10xHBSS (Gibco) + 45ml sterile H_2O + 1332µl sterile 7.5% BSA (Sigma Aldrich) + 1-3 drops of sterile 7.5% sodium bicarbonate solution (Gibco) until solution turns pink – filter sterilise with 25µ syringe filter and 50ml syringe

10xHBSS with $NaHCO_3$:
175mg $NaHCO_3$ + 50ml 10x HBSS without $NaHCO_3$ – use to make up 90% Percoll (45ml Percoll (GE Healthcare) + 5ml 10xHBSS with $NaHCO_3$)

Percoll preparations:

Percoll Concentration	For 10 ml (3 gradients)	
	90% Percoll	1x HBSS
81%	9ml	1ml
62%	6.9ml	3.1ml
55%	6.1ml	3.9ml

Appendix 2: EasySep™ murine blood neutrophil isolation protocol

1. Pellet whole leucocytes after red blood cell lysis
2. Resuspend in 150μl of RoboSep™ buffer (Stemcell technologies) and transfer to a 96 well plate
3. Add Rat Serum 7.5μl per 150μl sample
4. Add Enrichment cocktail 7.5μl per 150μl sample
5. Incubate at 4°C for 15 minutes
6. Wash cells by adding 100μl of buffer
7. Spin plate at 300g for 7 minutes
8. Aspirate off as much supernatant as possible (around 200-250μl)
9. Resuspend in initial volume (i.e. 150μl)
10. Add Biotin selection cocktail 7.5μl per 150μl sample
11. Incubate at 4°C for 15 minutes
12. Add magnetic particles (vortex beforehand) 20μl per 150μl sample
13. Incubate at 4°C for 10 minutes
14. Top up with buffer to a total of 250μl in the well (add 80μl buffer)
15. Incubate on a plate magnet for 10 minutes
16. Aspirate enriched cells carefully without disturbing pellet (around 200-250μl)
17. Pool (if appropriate) and top up to 500μl
18. Count (neat)
19. Top up with 10ml buffer to wash
20. Spin at 300g for 10 minutes at 4°C
21. Cells are ready to resuspend in media

Appendix 3: Lysis Buffers

Proteomic sample processing buffers:

stock	**lysis buffer**	**digest buffer**
20% SDS	1000 µL [4% final]	10 µL [0.1% final]
0.5M TCEP	100 µL [10 mM final]	-
1M TEAB	250 µL [50 mM final]	100 µL [50 mM final]
1M $CaCl_2$	-	2 µL [1 mM final]
H_2O	3650 µL	1888 µL

Neutrophil Protein Lysate Buffers

Laemmli Buffer		
0.5M Tris HCl pH 6.8	250ul	1.25ml
100% Glycerol	200ul	1ml
10% SDS	400ul	2ml
Complete inhibitors (Roche 04693159001) 1 tablet in 2ml	400ul	2ml
Phosphatase inhibitors (Roche 04906845001) 1 tablet in 500ul	100ul	500ul
H_2O	650ul	3.25ml
Final volume	**2ml**	**10ml**

Additional protease inhibitors were added to complete Laemmli buffer as follows:
Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1mM

SDS lysis buffer				
	Stock concentration	Target Concentration		To Add
		Molarity	%	
DTT	1M	0.2		308.5mg
SDS	20%		4%	2ml
Glycerol 100%	100%		20%	2ml
Tris-HCl pH 6.8	0.5M	0.1		2ml
Bromophenol Blue	2%		0.04%	0.1ml
Protease inhibitor cocktail				0.2ml
H_2O				3.7ml
Final Volume				**10ml**

Appendix 4: Immunoblot for protein expression

SDS-PAGE gels: For 1.5mm gel plates

Stacking Gel	**1 gel**	**2 gels**
Water	3ml	6ml
40% Acrylamide	620µl	1240µl
0.5M Tris pH 6.8	1260µl	2520µl
20% SDS	25µl	50µl
20% APS	50µl	100µl
TEMED	5µl	10µl

Resolving Gel	**8%**	**10%**	**12%**
Water	8ml	7.2ml	6.6ml
40% Acrylamide	3ml	3.8ml	4.4ml
1.5M Tris pH 8	3.8ml	3.8ml	3.8ml
20% SDS	75µl	75µl	75µl
20% APS	150µl	150µl	150µl
TEMED	6µl	6µl	6µl

Buffers for Immunoblot

10X Running Buffer	
Glycine	190g
Tris Base	30.3g
20% SDS	50ml
dH_2O	to 1 litre

1x Running buffer made with 100ml of 10x running buffer and 900ml of dH_2O

10X Transfer Buffer	
Glycine	145g
Tris Base	29g
dH_2O	to 800ml

1 X Transfer Buffer made with 100ml of 10X transfer buffer, 200ml methanol and 700ml dH_2O

10X TBS-Tween	
Tris-HCl 1M pH 8.0	100ml
NaCl	97.3g
Water	to 1000ml
Tween-20	5ml

1X TBS-Tween (0.05%) made with 100ml 10X TBS-Tween and 900ml dH_2O

Antibody concentrations

Target Protein	Host species	Source and Reference	Concentration
p38	Rabbit	Cell signaling technology #9212	1:2000
Elastase	Rat	Abcam Ab205670	1:1000
MMP9	Mouse	Abcam Ab58803	2.5:1000
MPO	Rabbit	Abcam Ab188211	1:1000
P70 S6 Kinase	Rabbit	Cell signaling technology #2708	1:500
Phospho-p70 S6 Kinase (Thr389)	Rabbit	Cell signaling technology #9234	1:500
Anti-rabbit immunoglobulin (HRP linked)	Goat	Dako p044801	1:2000
Anti-rat immunoglobulin (HRP linked	Goat	Cell signaling technology #7077	1:2000
Anti-mouse immunoglobulin (HRP linked)	Horse	Cell signaling technology #7076	1:2000

Appendix 5: RNA extraction using Acid-Phenol:Chloroform

1. Resuspend the cell pellet in 600μl of RNA lysis buffer
2. Pre-heat nuclease-free H_2O to 95C; allow wash solutions and filters to reach room temp
3. Add 1/10th volume of miRNA homogenate additive to the lysate to stabilise RNA and inactivate RNases (i.e. if lysate is 600μl, then add 60μl)
4. Vortex to mix
5. Incubate on ice for 10 minutes
6. Add a volume of Acid-Phenol:Chloroform equivalent to that of the initial lysate (i.e. 600μl). Withdraw the Acid-Phenol:Chloroform from the bottom phase of the tube (the top phase is a buffer solution)
7. Gently mix x3 then vortex for 60 seconds – ensure mixes well
8. Spin: 5 minutes, room temp, 13400rpm
9. Carefully remove the upper aqueous layer and transfer to a clean RNase-free Eppendorf
10. Note the volume removed
11. Add 1.25 volumes of 70% ethanol to the aqueous layer
12. Vortex
13. Pipette a maximum of 700μl of this aqueous layer/ethanol mixture onto a filter cartridge within a labelled collection tube
14. Spin: 15 seconds, room temp, 10000rpm
15. Discard the flow through
16. Repeat steps 12-14 if extra aqueous/ethanol mixture left
17. Pipette 700μl miRNA wash-solution 1 to the filter cartridge
18. Spin: 15 seconds, room temp, 10000rpm
19. Discard the flow through
20. Pipette 500μl miRNA wash-solution 2/3 to the filter cartridge
21. Spin: 15 seconds, room temp, 10000rpm
22. Discard the flow through
23. Pipette another 500μl miRNA wash-solution 2/3 to the filter cartridge
24. Spin: 15 seconds, room temp, 10000rpm
25. Discard the flow through then spin for further 60 seconds
26. Place filter cartridge into a clean fully-labelled collection tube
27. Pipette 30-50μl pre-heated H_2O onto the filter
28. Spin: 20-30 seconds, room temp, 13400rpm
29. Use nanodrop device to quantify RNA and assess purity

DNAse Treatment:

1. If sample contains>200ng/μl nucleic acid, dilute to <200ng/μl in RNase-free water
2. Add 1ul Turbo DNase per 40ul sample and 1ul 10x DNase buffer per 10ul sample
3. Incubate at 37°C for 20-30 minutes
4. Add DNase inactivation reagent 1ul per 10ul sample, mix then spin at 10,000G for 90 seconds at 4°C to pellet inactivation beads.

5. Repeat nanodrop to quantify being careful not to disturb/resuspend the pellet

Appendix 6: cDNA generation and TaqMan analysis

Reverse transcription PCR mastermix (Promega UK)

Reagent	Volume per sample
5x AMV buffer	8μl
10mM dNTPs	16μl
RNasin	1.2μl
Random primers	1.2μl
AMV-RT	1.2μl
Final volume	27.6μl

Primer probes

Target	Source and product reference	Exon location
Elane	Integrated DNA technologies Mm.PT.58.6682392.gs	4-5
MMP9	Integrated DNA technologies Mm.PT.58.10100097	8-9
MPO	Integrated DNA technologies Mm.PT.58.5251395	8-9
Egln1 (PHD2)	Thermo Fisher Scientific Mm00459770_m1	3-4
Egln2 (PHD1)	Thermo Fisher Scientific Mm00519067_m1	2-3
Egln3 (PHD3)	Thermo Fisher Scientific Mm00472200_m1	1-2
Actb	Integrated DNA technologies Mm.PT.39a.22214843.g	5-6

Appendix 7: Flow cytometry antibodies and panels

Lung Digest Flow Cytometry Antibody Panel

Antigen	Fluorophore	Concentration in mastermix	Source	Product code
CD11b	BV 421	1:400	Biolegend	101235
Ly6C	FITC	1:200	Biolegend	128005
Ly6G	PE	1:200	Biolegend	127607
SiglecF	PE CF594	1:800	BD	562757
CD64	APC	1:200	Biolegend	139305
CD45	AF 700	1:200	Biolegend	103128

Mouse Whole Blood Flow Cytometry Antibody Panel

Antigen	Fluorophore	Concentration in mastermix	Source	Product code
Ly6G	V450	1:200	Biolegend	127612
CD3	PE	1:200	Biolegend	100205
CD19	PE	1:200	Biolegend	152407
SiglecF	PE CF594	1:800	BD	562757
CD115	APC	1:200	Biolegend	135510
CD45	AF 700	1:200	Biolegend	103128
CD11B	APC Cy7	1:200	Biolegend	101225
CD62L	PE Cy7	1:200	Biolegend	104417

Additional Flow Cytometry Antibodies

Antigen	Fluorophore	Concentration in mastermix	Source	Product code
CSF2Ra	APC	7.5μl per test	Bio-techne	FAB6130A
CSF2Rb	PE	5μl per test	Bio-techne	FAB5492P
TNFRSF1	APC	1:100	Biolegend	113005
TNFRSF2	PE	1:100	Biolegend	113405
HIF-1α	PE	1:50	Cell signalling technology	59370S

Appendix 8: Chemotaxis assay plate plan

	+ve control	Chemokinesis control	Negative control	1nM KC	10nM KC	100nM KC	1000nM KC
Microplate well	25ul cells	29ul 100nM KC	29ul media	29ul of 1nM KC	29ul of 10nM KC	29ul of 100nM KC	29ul of 1000nM KC
Top of filter	nothing	25ul cells + 100nM KC	25ul cells	25ul cells	25ul cells	25ul cells	25ul cells

Each concentration of chemoattractant is run in duplicate for each sample.

Appendix 9: Kegg pathway enrichment

Term	Count	%	P Value	Pop Hits	Fold Enrichment
mmu03040:Spliceosome	125	2.10544	5.32E-44	133	2.57201
mmu00920:Sulfur metabolism	10	0.16844	0.00286	11	2.48783
mmu00030:Pentose phosphate pathway	27	0.45478	1.94E-08	30	2.46296
mmu00520:Amino sugar and nucleotide sugar metabolism	44	0.74112	1.30E-13	49	2.45737
mmu03050:Proteasome	40	0.67374	4.43E-12	45	2.43255
mmu00051:Fructose and mannose metabolism	29	0.48846	5.28E-08	34	2.33417
mmu04130:SNARE interactions in vesicular transport	28	0.47162	1.20E-07	33	2.32198
mmu00020:Citrate cycle (TCA cycle)	27	0.45478	2.72E-07	32	2.30902
mmu03030:DNA replication	29	0.48846	1.72E-07	35	2.26748
mmu00062:Fatty acid elongation	21	0.35371	3.11E-05	26	2.21035
mmu03018:RNA degradation	65	1.09483	1.43E-14	82	2.16927
mmu05134:Legionellosis	45	0.75796	3.90E-10	57	2.16049
mmu03015:mRNA surveillance pathway	75	1.26326	4.54E-16	96	2.13798
mmu00052:Galactose metabolism	25	0.42109	1.03E-05	32	2.13798
mmu00040:Pentose and glucuronate interconversions	28	0.47162	2.83E-06	36	2.12848
mmu00640:Propanoate metabolism	21	0.35371	7.86E-05	27	2.12848
mmu00511:Other glycan degradation	14	0.23581	0.00223	18	2.12848
mmu01200:Carbon metabolism	90	1.51592	5.70E-19	116	2.12324
mmu04666:Fc gamma R-mediated phagocytosis	65	1.09483	1.10E-13	84	2.11762
mmu04150:mTOR signaling pathway	45	0.75796	2.66E-09	59	2.08725
mmu05212:Pancreatic cancer	49	0.82533	8.82E-10	65	2.06299
mmu04662:B cell receptor signaling pathway	52	0.87586	5.87E-10	70	2.03292
mmu00010:Glycolysis / Gluconeogenesis	49	0.82533	2.09E-09	66	2.03173
mmu03013:RNA transport	126	2.12228	2.94E-23	170	2.02832
mmu05223:Non-small cell lung cancer	41	0.69058	1.07E-07	56	2.0036
mmu03430:Mismatch repair	16	0.2695	0.00252	22	1.99027
mmu05220:Chronic myeloid leukemia	52	0.87586	2.99E-09	72	1.97645
mmu01230:Biosynthesis of amino acids	56	0.94324	9.37E-10	78	1.96475
mmu05100:Bacterial invasion of epithelial cells	56	0.94324	9.37E-10	78	1.96475
mmu00620:Pyruvate metabolism	28	0.47162	3.26E-05	39	1.96475
mmu01130:Biosynthesis of antibiotics	153	2.57706	2.11E-25	214	1.95655
mmu04142:Lysosome	87	1.46539	1.68E-14	122	1.95152
mmu00860:Porphyrin and chlorophyll metabolism	29	0.48846	3.45E-05	41	1.93566
mmu04966:Collecting duct acid secretion	19	0.32003	0.00139	27	1.92577
mmu00053:Ascorbate and aldarate metabolism	19	0.32003	0.00139	27	1.92577
mmu04621:NOD-like receptor signaling pathway	39	0.6569	1.76E-06	56	1.90586
mmu04664:Fc epsilon RI signaling pathway	47	0.79165	1.66E-07	68	1.89149
mmu04141:Protein processing in endoplasmic reticulum	116	1.95385	1.56E-17	168	1.88957
mmu01212:Fatty acid metabolism	35	0.58952	1.13E-05	51	1.87807
mmu01210:2-Oxocarboxylic acid metabolism	13	0.21897	0.01645	19	1.87242
mmu04370:VEGF signaling pathway	41	0.69058	1.90E-06	60	1.87002
mmu05221:Acute myeloid leukemia	38	0.64005	6.37E-06	56	1.85699
mmu00071:Fatty acid degradation	33	0.55584	3.84E-05	49	1.84303
mmu00280:Valine, leucine and isoleucine degradation	37	0.62321	1.17E-05	55	1.841
mmu05132:Salmonella infection	52	0.87586	1.90E-07	78	1.82441
mmu04144:Endocytosis	185	3.11605	1.16E-24	278	1.82113
mmu05214:Glioma	43	0.72427	3.63E-06	65	1.81038
mmu05211:Renal cell carcinoma	44	0.74112	3.63E-06	67	1.79718
mmu05230:Central carbon metabolism in cancer	42	0.70743	6.53E-06	64	1.79591
mmu04722:Neurotrophin signaling pathway	80	1.34748	1.71E-10	122	1.7945
mmu04210:Apoptosis	39	0.6569	2.09E-05	60	1.7788
mmu04910:Insulin signaling pathway	90	1.51592	5.23E-11	140	1.75925
mmu05169:Epstein-Barr virus infection	138	2.32441	2.60E-16	215	1.75653
mmu05142:Chagas disease (American trypanosomiasis)	66	1.11167	3.23E-08	103	1.75356
mmu05210:Colorectal cancer	41	0.69058	2.02E-05	64	1.75315
mmu04919:Thyroid hormone signaling pathway	72	1.21273	9.81E-09	113	1.74369
mmu04720:Long-term potentiation	42	0.70743	1.97E-05	66	1.74148
mmu00480:Glutathione metabolism	35	0.58952	1.15E-04	55	1.74148
mmu04380:Osteoclast differentiation	80	1.34748	1.67E-09	126	1.73754
mmu01040:Biosynthesis of unsaturated fatty acids	17	0.28634	0.01339	27	1.72306
mmu04070:Phosphatidylinositol signaling system	61	1.02745	3.06E-07	97	1.72097
mmu00562:Inositol phosphate metabolism	44	0.74112	1.86E-05	70	1.72016
mmu04962:Vasopressin-regulated water reabsorption	27	0.45478	0.00117	43	1.71834
mmu05020:Prion diseases	20	0.33687	0.00702	32	1.71039

Term	Count	%	P Value	Pop Hits	Fold Enrichment
mmu05133:Pertussis	46	0.7748	1.73E-05	74	1.70114
mmu04071:Sphingolipid signaling pathway	77	1.29695	1.46E-08	124	1.69935
mmu04012:ErbB signaling pathway	54	0.90955	2.91E-06	87	1.69859
mmu00630:Glyoxylate and dicarboxylate metabolism	18	0.30318	0.01256	29	1.69859
mmu03060:Protein export	18	0.30318	0.01256	29	1.69859
mmu04922:Glucagon signaling pathway	62	1.0443	4.93E-07	100	1.6967
mmu04660:T cell receptor signaling pathway	64	1.07799	4.57E-07	104	1.68407
mmu05213:Endometrial cancer	32	0.53899	5.72E-04	52	1.68407
mmu00980:Metabolism of xenobiotics by cytochrome P450	39	0.6569	1.59E-04	64	1.66763
mmu05140:Leishmaniasis	39	0.6569	1.59E-04	64	1.66763
mmu00900:Terpenoid backbone biosynthesis	14	0.23581	0.03996	23	1.66577
mmu05152:Tuberculosis	107	1.80226	9.20E-11	176	1.66374
mmu04145:Phagosome	105	1.76857	2.60E-10	174	1.65141
mmu00270:Cysteine and methionine metabolism	24	0.40424	0.00544	40	1.64197
mmu04920:Adipocytokine signaling pathway	43	0.72427	1.28E-04	72	1.63437
mmu05231:Choline metabolism in cancer	60	1.01061	5.56E-06	101	1.62571
mmu00500:Starch and sucrose metabolism	19	0.32003	0.01782	32	1.62487
mmu04152:AMPK signaling pathway	75	1.26326	4.23E-07	127	1.61611
mmu04146:Peroxisome	49	0.82533	5.85E-05	83	1.61559
mmu00983:Drug metabolism - other enzymes	30	0.50531	0.00238	51	1.60978
mmu03010:Ribosome	85	1.4317	1.01E-07	145	1.60422
mmu04915:Estrogen signaling pathway	57	0.96008	2.35E-05	98	1.59171
mmu04721:Synaptic vesicle cycle	36	0.60637	0.00105	62	1.589
mmu04912:GnRH signaling pathway	51	0.85902	7.77E-05	88	1.58599
mmu04668:TNF signaling pathway	63	1.06114	1.07E-05	109	1.58172
mmu04120:Ubiquitin mediated proteolysis	82	1.38117	4.20E-07	142	1.5803
mmu05161:Hepatitis B	84	1.41486	3.69E-07	146	1.57449
mmu04960:Aldosterone-regulated sodium reabsorption	23	0.3874	0.01291	40	1.57356
mmu05164:Influenza A	98	1.65067	4.47E-08	171	1.56835
mmu04931:Insulin resistance	63	1.06114	1.58E-05	110	1.56734
mmu05145:Toxoplasmosis	64	1.07799	2.16E-05	113	1.54994
mmu04611:Platelet activation	74	1.24642	5.04E-06	131	1.54588
mmu04914:Progesterone-mediated oocyte maturation	49	0.82533	2.81E-04	87	1.54131
mmu05203:Viral carcinogenesis	130	2.18966	1.05E-09	231	1.54009
mmu04917:Prolactin signaling pathway	41	0.69058	0.00106	73	1.537
mmu04622:RIG-I-like receptor signaling pathway	38	0.64005	0.0019	68	1.52929
mmu04540:Gap junction	48	0.80849	4.34E-04	86	1.52741
mmu04730:Long-term depression	34	0.57268	0.0037	61	1.52533
mmu04810:Regulation of actin cytoskeleton	119	2.00438	1.48E-08	214	1.52176
mmu04520:Adherens junction	40	0.67374	0.00162	72	1.52034
mmu04114:Oocyte meiosis	61	1.02745	7.98E-05	110	1.51758
mmu04620:Toll-like receptor signaling pathway	56	0.94324	1.66E-04	101	1.51733
mmu00510:N-Glycan biosynthesis	27	0.45478	0.01302	49	1.50793
mmu05012:Parkinson's disease	82	1.38117	5.71E-06	149	1.50606
mmu05014:Amyotrophic lateral sclerosis (ALS)	28	0.47162	0.01194	51	1.50246
mmu04066:HIF-1 signaling pathway	57	0.96008	2.15E-04	104	1.49988
mmu05222:Small cell lung cancer	46	0.7748	0.001	84	1.49862
mmu00190:Oxidative phosphorylation	76	1.28011	1.79E-05	139	1.49628
mmu04932:Non-alcoholic fatty liver disease (NAFLD)	85	1.4317	8.83E-06	157	1.48161
mmu04670:Leukocyte transendothelial migration	65	1.09483	1.57E-04	121	1.47008
mmu05215:Prostate cancer	47	0.79165	0.00174	88	1.4616
mmu04650:Natural killer cell mediated cytotoxicity	56	0.94324	6.09E-04	105	1.45953
mmu05010:Alzheimer's disease	94	1.58329	8.04E-06	177	1.45335
mmu00982:Drug metabolism - cytochrome P450	35	0.58952	0.00883	66	1.45124
mmu05160:Hepatitis C	72	1.21273	1.20E-04	136	1.4488
mmu05168:Herpes simplex infection	110	1.85279	1.61E-06	208	1.44725
mmu03420:Nucleotide excision repair	23	0.3874	0.04602	44	1.4305
mmu04068:FoxO signaling pathway	70	1.17905	2.55E-04	134	1.42958
mmu05162:Measles	71	1.19589	2.34E-04	136	1.42868
mmu05146:Amoebiasis	61	1.02745	7.20E-04	117	1.42678
mmu00310:Lysine degradation	27	0.45478	0.03171	52	1.42094
mmu05016:Huntington's disease	102	1.71804	1.76E-05	198	1.40977
mmu04921:Oxytocin signaling pathway	81	1.36433	1.76E-04	158	1.40295
mmu05205:Proteoglycans in cancer	104	1.75173	1.95E-05	203	1.40201
mmu04330:Notch signaling pathway	25	0.42109	0.04952	49	1.39623
mmu04064:NF-kappa B signaling pathway	49	0.82533	0.00582	97	1.38242
mmu04612:Antigen processing and presentation	41	0.69058	0.01493	82	1.36831
mmu00561:Glycerolipid metabolism	29	0.48846	0.04364	58	1.36831
mmu01100:Metabolic pathways	637	10.7293	9.76E-27	1278	1.36403

Term	Count	%	P Value	Pop Hits	Fold Enrichment
mmu00240:Pyrimidine metabolism	51	0.85902	0.00792	103	1.35502
mmu04728:Dopaminergic synapse	66	1.11167	0.00277	134	1.34789
mmu04010:MAPK signaling pathway	124	2.0886	4.22E-05	253	1.34127
mmu04110:Cell cycle	58	0.97692	0.01857	124	1.28003
mmu00230:Purine metabolism	82	1.38117	0.00943	179	1.25365
mmu04062:Chemokine signaling pathway	89	1.49907	0.00901	196	1.24265
mmu04261:Adrenergic signaling in cardiomyocytes	67	1.12852	0.03446	150	1.22236
mmu04510:Focal adhesion	90	1.51592	0.03061	207	1.18983
mmu04015:Rap1 signaling pathway	93	1.56645	0.02839	214	1.18928
mmu05166:HTLV-I infection	118	1.98754	0.02974	278	1.16159

Appendix 10: Labelled proteins

Protein IDs	Protein names	Gene names	Peptides	Unique peptides	H/L Ratio 1	H/L Ratio 2	H/L Ratio 3	H/L Ratio 4
O09172	Glutamate-cysteine ligase regulatory subunit	Gclm	3	3	1.579	2.133	1.493	NaN
O54824	Interleukin-16	Il16	19	19	NaN	0.105	0.183	NaN
O89053	Coronin-1A	Coro1a	46	44	NaN	0.014	0.047	0.034
P01027	Complement C3	C3	83	73	0.026	0.048	NaN	NaN
P01887	Beta-2-microglobulin	B2m	6	6	0.328	0.463	0.501	NaN
P05064	Fructose-bisphosphate aldolase A	Aldoa	50	39	0.142	0.171	0.199	0.172
P06151	L-lactate dehydrogenase A chain	Ldha	28	26	0.099	0.070	0.082	0.062
P07356	Annexin A2	Anxa2	47	47	0.031	0.060	0.054	NaN
P08752	Guanine nucleotide-binding protein G	Gnai2;Gnai1	26	18	0.095	0.043	0.090	0.058
P08905	Lysozyme C-2	Lyz2	15	8	0.007	0.013	0.020	NaN
P09411	Phosphoglycerate kinase 1	Pgk1	46	46	0.158	0.137	0.058	0.028
P09528	Ferritin heavy chain	Fth1	18	18	1.274	2.055	2.514	1.563
P10126	Elongation factor 1-alpha 1	Eef1a1	33	22	0.025	NaN	0.066	NaN
P10649	Glutathione S-transferase Mu 1	Gstm1	26	22	0.052	0.099	0.083	NaN
P10810	Monocyte differentiation antigen CD14	Cd14	5	5	1.149	1.861	NaN	1.735
P10852	4F2 cell-surface antigen heavy chain	Slc3a2	2	2	1.837	2.489	2.045	NaN
P11835	Integrin beta-2	Itgb2	69	68	0.045	0.103	0.113	0.048
P13020	Gelsolin	Gsn	60	36	0.043	0.081	0.094	NaN
P15379	CD44 antigen	Cd44	4	4	0.247	0.259	0.363	NaN
P16110	Galectin-3	Lgals3	12	12	0.147	0.230	0.166	0.112
P16858	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	40	40	0.048	NaN	0.058	0.045
P17182	Alpha-enolase	Eno1	55	52	0.043	0.095	0.031	0.051
P17742	Peptidyl-prolyl cis-trans isomerase A	Ppia	23	23	0.228	0.096	0.127	NaN
P17751	Triosephosphate isomerase	Tpi1	18	18	0.104	0.266	0.127	0.147
P18242	Cathepsin D	Ctsd	7	7	0.265	0.511	0.455	0.337
P20029	78 kDa glucose-regulated protein	Hspa5	51	50	0.187	0.247	0.704	NaN
P24527	Leukotriene A-4 hydrolase	Lta4h	55	55	0.160	0.034	NaN	NaN
P26041	Moesin	Msn	62	46	0.146	0.225	0.218	0.095
P27005	Protein S100-A8	S100a8	11	11	0.017	NaN	0.034	NaN
P29351	Tyrosine-protein phosphatase non-receptor type 6	Ptpn6	31	31	0.168	0.232	NaN	NaN
P29391	Ferritin light chain	Ftl1;Ftl2	16	16	6.162	6.615	5.124	3.798
P31725	Protein S100-A9	S100a9	23	23	0.024	0.004	0.029	0.015
P34884	Macrophage migration inhibitory factor	Mif	3	3	0.521	0.707	NaN	NaN
P35700	Peroxiredoxin-1	Prdx1	12	11	NaN	0.784	0.533	0.611
P37040	NADPH-cytochrome P450 reductase	Por	15	15	0.400	0.502	NaN	NaN
P51150	Ras-related protein Rab-7a	Rab7a	13	13	0.416	0.215	0.196	0.115
P54987	Cis-aconitate decarboxylase	Irg1	8	8	1.607	1.781	1.370	1.214
P58058	NAD kinase	Nadk	5	5	0.771	0.648	0.811	NaN
P60335	Poly(rC)-binding protein 1	Pcbp1	17	15	0.240	0.240	NaN	NaN
P62962	Profilin-1	Pfn1	20	9	0.019	0.015	NaN	NaN
P63017	Heat shock cognate 71 kDa protein	Hspa8	55	49	NaN	0.018	0.023	NaN
P63101	14-3-3 protein zeta/delta	Ywhaz	29	20	0.062	0.082	NaN	0.040
P70460	Vasodilator-stimulated phosphoprotein	Vasp	29	29	0.231	0.259	0.267	0.127
P84228	Histone H3	Hist1h3b; Hist1h3a	23	23	NaN	NaN	0.062	0.014
P99029	Peroxiredoxin-5, mitochondrial	Prdx5	22	22	0.040	0.085	0.049	0.041
Q05144	Ras-related C3 botulinum toxin substrate 2	Rac2	22	14	NaN	0.073	0.067	0.048
Q09014	Neutrophil cytosol factor 1	Ncf1	31	31	0.064	0.205	0.224	NaN
Q61207	Prosaposin	Psap	9	9	NaN	0.613	0.599	1.319
Q61233	Plastin-2	Lcp1	56	56	0.100	0.152	0.106	0.062
Q61362	Chitinase-3-like protein 1	Chi3l1	15	15	NaN	0.286	0.778	0.590
Q61792	LIM and SH3 domain protein 1	Lasp1	23	23	NaN	0.104	0.096	NaN
Q62426	Cystatin-B	Cstb	6	6	0.335	NaN	0.907	NaN
Q8R460	Interleukin-36 gamma	Il36g	4	4	0.420	0.613	NaN	NaN
Q8VDD5	Myosin-9	Myh9	301	253	0.047	0.064	0.055	0.024
Q91VW3	SH3 domain-binding glutamic acid-rich-like protein 3	Sh3bgrl3	9	9	0.031	0.053	0.078	NaN
Q93092	Transaldolase	Taldo1	35	35	0.063	0.118	0.101	0.089
Q99JI6	Ras-related protein Rap-1b	Rap1b	11	3	NaN	0.206	0.169	NaN
Q9DBJ1	Phosphoglycerate mutase 1	Pgam1	30	30	0.391	0.185	0.108	NaN
Q9DCD0	6-phosphogluconate dehydrogenase, decarboxylating	Pgd	36	36	0.055	0.056	0.046	NaN
Q9JHK5	Pleckstrin	Plek	16	16	0.170	0.688	NaN	NaN
Q9JKF	Ras GTPase-activating-like protein IQGAP1	Iqgap1	81	80	0.197	0.141	0.193	0.109
Q9R0P3	S-formylglutathione hydrolase	Esd	11	11	0.385	0.626	NaN	NaN

Appendix 11: Additional Materials

Product	Source	Product number
IgM ELISA	Abcam	Ab133047
Albumin ELISA	Abcam	Ab108792
GM-CSF ELISA	Abcam	Ab201276
Glutamine detection assay	Abcam	Ab197011
MMP9 ELISA	Bio-Techne	MMPT90
EnzCheck Elastase Activity Assay kit	Invitrogen	E12056
EnzCheck MPO Activity Assay kit	Invitrogen	E33856
Shandon Kwik-Diff stains	Thermo Scientific	9990700
DPX mountant	Sigma	06522-100ml
LAMP1 Antibody	Abcam	Ab25630
Goat anti-mouse IgG H&L AF647	Abcam	Ab150115
Fixation/Permeabilisation Solution kit	BD Biosciences	54714
Texas red 70kDa dextran	Invitrogen	D1864
Texas red BSA	Invitrogen	A23017
FITC-BSA	Invitrogen	A23015
DQ-Green BSA	Invitrogen	D12050
AF488 E coli	Invitrogen	E13231
CountBright beads	Invitrogen	C36950
Prolong gold w DAPI	Invitrogen	P36931
Cellmask far red	Invitrogen	H32721
H2DCFDA	Invitrogen	C6827
Red blood cell lysis buffer	Sigma	R7757-100ml
TCEP	Sigma	68957
EZQ	Invitrogen	R33200
LysC	Promega	VA1170
Trypsin	Promega	V5280
CBQCA	Invitrogen	C6667
LPS	Sigma	L7018
Red blood cell lysis buffer	Biolegend	420301
EasySep kit	Stemcell technologies	19762
Glucose assay	Biovision	K606
13C6 Lysine	Cambridge Isotope Laboratories	CLM-2247-H
13C6 15N4 Arginine	Cambridge Isotope Laboratories	CLNM-539-H
13C5 Glutamine	Cambridge Isotope Laboratories	CLM-1822-H
Percoll	GE Healthcare	17-0891-01
ChemoTx plates	Neuro Probe	101-5
Glycogen assay	Sigma	MAK016
Chloroquine	Sigma-Aldrich	C6628
E64	Sigma-Aldrich	E3132
Robosep buffer	Stemcell technologies	20104
BCA assay kit	Thermo Scientific	23225